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Cytogenetic studies: Premature chromosome condensation (PCC) in peripheral blood lymphocytes cultures and skin warts lesions cultures induced by Papillomavirus spontaneous infection in bovines

Estudos citogenéticos: condensação cromossômica prematura (PCC) em culturas de linfócitos do sangue periférico e culturas de lesões de verrugas cutâneas induzidas pela infecção espontânea do papilomavírus em bovinos

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Abstract

Papillomavirus is a dermotropic oncogenic DNA virus which induces benign and malignant lesions described in a wide variety of hosts. Evidences suggest that the virus is species and tissue specific. However several reports have shown the development of lesions in phylogenetically close hosts. We analyzed a total of 30 blood and tumors samples from bovines affected cutaneous papillomatosis lesions. Our study reported premature chromosome condensation (PCC) in peripheral blood lymphocytes cultures and in skin warts cells cultures, and we detected epithelial cellular hyperproliferation confirmed by histological tissues processing. The morphology of the PCC reflects the cell cycle phase-specific exhibiting levels of the prematurely condensed chromatin. These results indicated a failed in the cellular control mechanism with disruption of mitotic checkpoints induced by virus spontaneous infection.

Key Words: Pappilomavirus (PV) – PCC – Bovine Infection.

Resumo

O papilomavírus é um vírus DNA oncogênico dermotrópico que induz a lesões benignas e malignas descrita em uma ampla variedade de hospedeiros. Evidências sugerem que o vírus é espécie e tecido específico. No entanto, vários relatos tem mostrado o desenvolvimento de lesões em hospedeiros filogeneticamente próximos. Foram analisadas um total de 30 amostras de sangue e tumores de bovinos afetados por lesões papilomatosas cutâneas. Nosso estudo relatou a condensação cromossômica prematura (PCC) em culturas de linfócitos do sangue periférico e em culturas de verrugas cutâneas, detectando a hiperproliferação celular epitelial, confirmada pelo processamento de tecidos histológicos. A morfologia da PCC reflete a fase do ciclo celular, exibindo níveis específicos da cromatina condensada prematuramente. Estes resultados indicaram uma falha no mecanismo de controle celular, com o rompimento de barreiras mitóticas, induzidas pela infecção espontânea do vírus.

Palavras-chave: Papilomavírus – PCC - Infecção em Bovinos.

INTRODUCTION

Papillomaviruses (PV) have been recently classified in a large family of species-specific viruses called *Papillomaviridae*, and a new system of genera based in a wide variety of host species phylogenetically relationships has been described (HOWLEY & LOWY., 2001). PVs are closed-circular double-stranded DNA genomes that infect the epithelial basal cells and the activities of the viral proteins induced the hyperproliferation in the cellular pathways causing to benign and malignant lesions.

Experimental studies research the latency period of the tumors in different levels about carcinogenesis mechanism. Firstly, initiation stage, which is when the cancer agent induced mutations and velocity alteration in the cell division and the next stage is the promotion, which in this process, the cells changed itself and happens abnormal development with decontrol proliferation of the cancerous cells until the tumor clinic formation (FILHO & GTTÁS., 2001).

The malign transformation happens after a long latency period, when the viral genome is integrated to the genome of the host cell. The place of the viral integration with the chromosomes in the malign lesions

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is random, but there is predilection for closely regions related to the cellular oncogenes (HOWLEY & LOWY., 2001).

In human, cancer development can be available trough mutagenous tests like peripheral lymphocytes cytogenetic analyses and lymphocytes micronucleus research or epithelial desquamations. Mutations or chromosomal aberrations involved modifying in the structure and numbers of the chromosomes that can be identifying in the optic microscopic (FILHO & GTTÁS., 2001).

In animals, exposition of cattle in bracken fern (*Pteridium aquilinum*) ingestion can be considered a cancer agent which interferes in the early or late levels carcinogenesis process acting like a cofactor in papillomavirus infection. Bracken fern contains immunosuppressant and mutagen substances that probably induce chromosomal abnormalities (LEAL et al., 2003). In bovines experimentally infected, the increase of chromosomal aberrations was observed associated to the transmission of *bovine papillomavirus* (BPV), suggesting that the virus can be transmitted horizontally by the blood and associated the clastogenic action of BPV in the absence of bracken fern ingestion (STOCCO DOS SANTOS et al., 1998).

Lioi et al. found in cytogenetic analysis, chromosome aberrations described as: breakages, chromatid breaks, fragments and chromosomal rearrangements. These animals had bladder cancer and molecular research detected of DNA sequences of the papillomavirus type 2, *Bovine papillomavirus* 2 (BPV-2). The correlation of the data suggested that the BPV-2 in association with the bracken fern, act synergistically in the production of genome instability (LIOI et al., 2004).

The viruses have the ability to penetrate cells by different mechanisms leading to chromosomal instability and some of them contribute to the development of cancer cells (DUELLI & LAZEBNIK, 2007). In general, mitotically active cutaneous and mucosal epithelial cells in all papillomaviruses are capable of maintaining their viral genomes with low-copy-number extrachromosomal factors in persistent infections (Mc PHILLIPS et al., 2006). Papillomavirus genomes do not contain centromeric sequences and the mechanism of segregation genome and chromosomal attachment has been studied. To the initiation of viral DNA replication, viral E2 protein induces the formation of the complex with the viral E1 protein and also functions as a transcriptional regulator of viral gene expression (Mc PHILLIPS et al., 2006).

Others proteins like open reading frames (ORFs) E5, E6 and E7 are also involved as crucial factors in the development of the cancer (MANSUR & ANDROPHY., 1993). These genes interfering with the normal epithelial differentiation and induce a failed in the cellular control of the viral transcription (MANSUR & ANDROPHY., 1993). The viral oncoproteins E6 and E7 to bind a complex and degradation of the tumor suppressor protein, p53, and retinoblastoma tumor suppressor protein, pRb, respectively. These proteins are consistently expressed in cells infected by papillomavirus (THOMPSON et al., 1997).

ATR enzyme is classified in the Kinase family and it is able to prevent the cellular induction to PCC by *checkpoint* G1 control. But, the cancerous cells and the p53 function lost, induce the ATR inhibition and consequently interfer in the *checkpoint* G1 inducing the formation PCC cells. After ATR had been recruited to cellular replication, it signalizes which the DNA synthesis not is completely ready and that the chromatin condensation cannot be starting. So, the *checkpoint* G1 is interrupted by cyclins over expression and induces the cells to entry in the S phase of cellular cycle where the cellular damage not is repair (NGHIEM et al., 2001).

Viral oncogenes expression that inactive the tumor suppressor proteins functions is directly associated with chromosomal aberrations formation (CHANG et al., 1997) and in these cells infected by papillomavirus chromosomal abnormalities related to checkpoint G2 cellular cycle alterations are observed (COURSEN et al., 1997).

PCC applications have been used for studies of chromosome analyzing several chromatin events. Drug-induced PCC technique by protein phosphatase inhibitors, or virus-mediated PCC method by interphase cell fusion to mitotic cells (GOTOH & DURANTE, 2006).

To investigate the failures in the control mechanisms, many studies have demonstrated disruption of mitotic checkpoints under the influence of factors in mitotic cells through of the use of several drugs chemically induced S-phase PCC and sometimes examined *in vitro* the frequency and sensitivity of these drugs treatment in different cell lines expressing mutant gene or cells infected with the control vector. The interaction of theses factors is important for the maintenance of viral genome (Mc PHILLIPS et al., 2006; THOMPSON et al., 1997).

Recently, chemical procedures have been developed to induce the PCC using protein phosphatase inhibitors, such as caffeine (THOMPSON et al., 1997), methyl methanosulfanate (MMS) (GARCIA et al., 2001), citocalasin B (TRIMBORN et al., 2004), endothal and cantharidine (GOTOH, 1996 apud GOTOH & DURANTE, 2006), and okadaic acid (GOTOH and DURANTE, 2006). Injuries in DNA cellular produced by camptothecin (CPT) induce premature chromosome condensation in the peripheral blood lymphocytes in humans (MOSESSO et al., 1999 apud GARCIA et al., 2001). The PCC can also be chemically induced by calyculin A when substitutes the use of colcemid in a conventional protocol (GOTOH & DURANTE, 2006) and when inserted in the peripheral blood lymphocytes of patients with lung cancer submitted radiotherapy treatment (LEE et al., 2004).

In the present study, we report the exacerbate proliferation in differentiated cells investigated by histopathology and cytogenetic analysis researching the premature chromosomes and chromatin constitution in peripheral blood lymphocytes cultures and tumors cells cultures (skin warts lesions) obtained from animals affected by bovine cutaneous papillomatosis.

MATERIAL AND METHODS

Selection of the animals

Ten bovines (*Bos taurus taurus* x *Bos taurus indicus*), both sexes, age varying among five months to five years, from North of Rio de Janeiro State, Brazil, were selected. All animals were verified presenting cutaneous papillomatosis lesions with skin warts signs in different anatomic regions. Experimental animals were shown to be free of chronic enzootic haematuria and not feeding on bracken fern pastures. Clinical evaluation was performed and blood and epithelial lesions samples from each animal were collected.

Blood and skin warts samples

Blood was collected approximately 5 mL using heparin vacutainer tube or heparinized syringe by the jugular or mammary vein. After collected, the tube was homogenized and the samples were cooled at 4-8ºC to use for lymphocyte culture. Under aseptic conditions, tumors tissues were extracted by epithelial biopsy from individual affected bovines. We choose lesions presenting different kinds of morphology. One of the fragments was immersed in RPMI medium cultured to tumors cultures analysis and another part of lesion was fixed in 10% phosphate-buffered formalin solution. All the samples were stored in a refrigerated thermal box and transported immediately to the laboratory.

Histopathology examination

Cutaneous papillomatosis lesions samples were fixed for tissue processing by histopathology in the Morphology and Pathological Anatomy Department, Animal Health Laboratory for histotecnic application. The tissue sections were routinely embedded in paraffin, cut at 4 mm, and stained with hematoxylin and eosin (H&E).

Cytogenetic analysis

Procedure for long term culture to implant of peripheral blood lymphocytes cultures and short term culture to implant fragments from tissues biopsy were obtained. The lymphocytes cultures consisted of 6 mL RPMI 1640 medium, 2 mL bovine fetal serum and 0,2 mL phytohaemagglutinin added in the total blood samples. The material was put in incubation at 37º C during 71 hours. After this time, 0,2 mL colchicin was added in the bottle of cellular cultivation and 72 hours finished. The material was transferred to falcon tubes and centrifuged a 1000 rpm for 10 minutes. Approximately 5 mL hypotonic solution KCl 0,075 M in 37ºC was added. The material was fixed in methanol: acetic acid for three times.

To implant skin warts cells from cutaneous biopsy, the fragments were cultured in 3 mL of RPMI 1640 medium, 0,2 mL of colchicin (16mg/mL) and 1mL of bovine fetal serum were added. After 40 minutes in 37ºC, the material was centrifuged and 10 mL of hypotonic solution KCl 0,075 M was added. The material was fixed three times in methanol: acetic acid (3:1). The slides were stained with Giemsa 3% in phosphate buffer, pH 6.8 and analyzed in the optical microscope.

Statistical analysis

The percentage of metaphases and proportion of PCC cells in lymphocytes of bovines with cutaneous papillomatosis disease was evaluated by Wilcoxon test. The cytogenetic data were also analysed using Wilcoxon test for the comparison among the amount of PCC cells in peripheral blood lymphocytes cultures and PCC cells in skin warts lesions cultures.

RESULTS

Clinical evaluation revealed some tumors presenting circumscribed and irregular aspects, occasionally ulcerated with coloration and differentiated morphologies, adhered the skin and flat, or pedunculated form and intermediate neoformations similar mixed lesions in several anatomic regions (Figure 1).

The histopathology exams demonstrated the viral cytopatic effect in papilliforms lesions independent of the morphology (Figure 2). Histologically, the tumors were characterized by fibroblastic proliferation exhibiting an infiltrative growth at the interface with normal tissue, epithelial hyperplasia, presence of koilocytes and keratohyalin-like granules in the granular layer (Figures 3 and 4).

In the conventional Giemsa staining, the cells analyzed was classified accordance with the numbers of the chromosomes, presence of PCC and the occurrence of possible rearrangements after the identification of each type of cultures (Figure 5).

In lymphocytes cultures were analysed 276 configurations of chromosome pulverization and in skin warts cells cultures were detected approximately 1817 images similar to premature chromosome condensation showing several stages of chromatin condensation. In some images the chromatin was observed no condensed and in others the interphase chromatin was condensed prematurely showed mitotic-like chromosomes. We observed cells with a single chromatid per chromosome typical from G1 phase of the interphase, cells from S phase exhibiting a “pulverized” appearance like several fragmented chromosomes (Figures 6 and 7), with both single and double chromatids and finally cells from G2 phase exhibiting two chromatids per chromosome. These images represent interphase cells, which has suffered a fusion with mitotic cells resulting in different degrees of

chromosome condensation according to the phase of the cellular cycle (Figures 8 and 9).

These observations are in accordance with Premature Chromosome Condensation (PCC) observed previously in other tumors types which reflects a high proliferative potential index (PPI) in uncontrolled proliferation of the cancerous cells. The frequency of cells in the interphase is resulting of cellular cycle interruption before the decontrolled proliferation of the cancerous cells infected by virus capable to inhibit the repairer function of protein kinase ATR.

Considering the total number of cells in lymphocytes culture analysed, the percentage of metaphases was 64,08% ± 26,15% (CV = 0.41) and the proportion of PCC was 35,92% ± 26,15% (CV = 0.73). Statistically, was not found significant differences between the proportions of metaphases and PCC cells, with p-value of 0,173.

In addition, the count of PCC cells in lymphocytes and frequency of PCC cells in the skin warts lesions were also analysed. Statistical comparison revealed that there is significant difference, by the Wilcoxon test with p-value equal to 0,005, between the medium number of PCC cells in lymphocytes (27,60± 33,38, CV = 1.21) and the number of PCC cells in the warts lesions cultures (181,70 ± 190,33, CV = 1.05). On the other hand, the number of PCC in lymphocytes not depend on the number of PCC in the warts lesions, because there isn´t correlation between the both variables (fig.10).

DISCUSSION AND CONCLUSION

The phenomenon "Premature Chromosome Condensation" or PCC was described by the first time in decade 70 by Rao and Johnson using Sendai virus and this event that follow fusion between mitotic and interphase cells has also been referred to as

Figure 1. Skin warts lesions from bovines cutaneous papillomatosis.

Figure 2. Basophilic intranucelar inclusion bodies in granulous layer indicated cytopatic effect of viral infection. H&E, 10x.

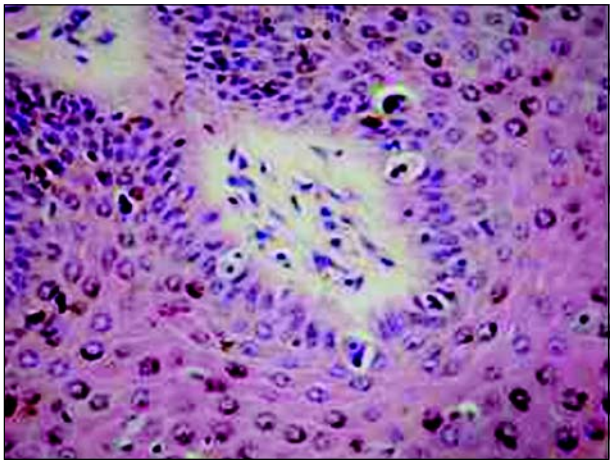


Figure 3. Hyperproliferation in epidermal layer with abundance inflammatory cells specifically lymphocytes and plasmocytes associated cells. H&E, 40x.

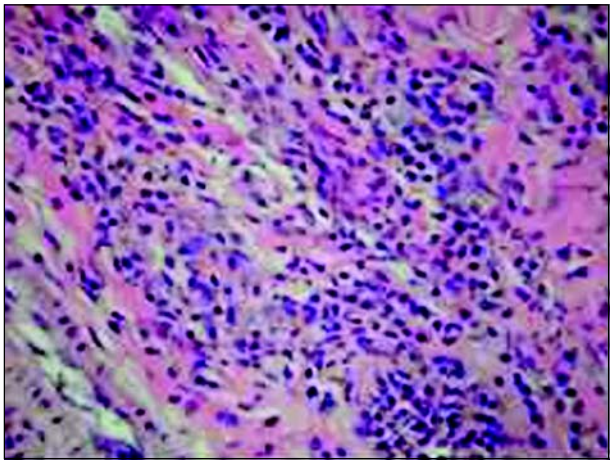


Figure 4. Hyperkeratosis and hypergranulosis by presence of keratohyalin granules in the granular layer of epithelial tissue. H&E, 10x.

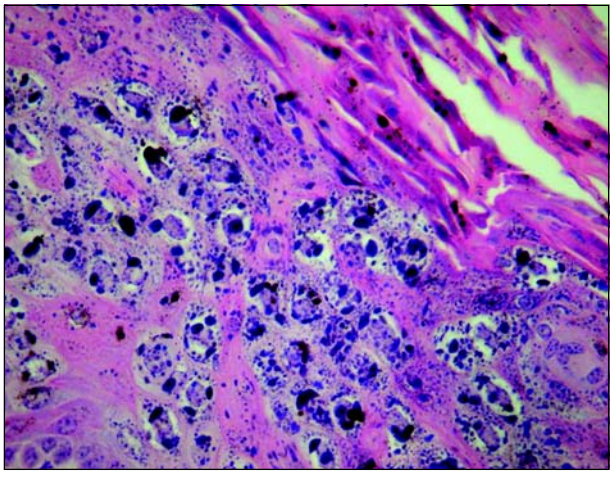


Figure 5. PCC cells similar prophase detected in peripheral blood lymphocytes cultures from bovines with cutaneous papillomatosis lesions.

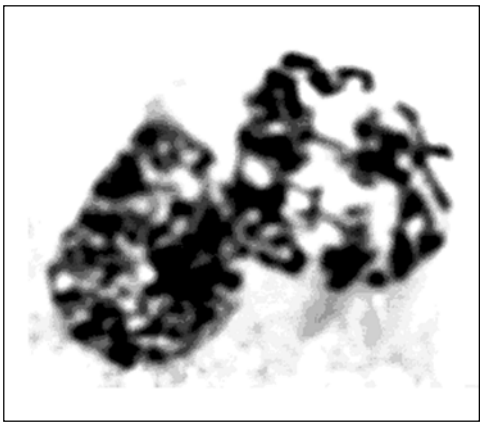


Figure 6. Premature chromosomes condensation (PCC) exhibiting appearance pulverized in skin warts lesions cultures.

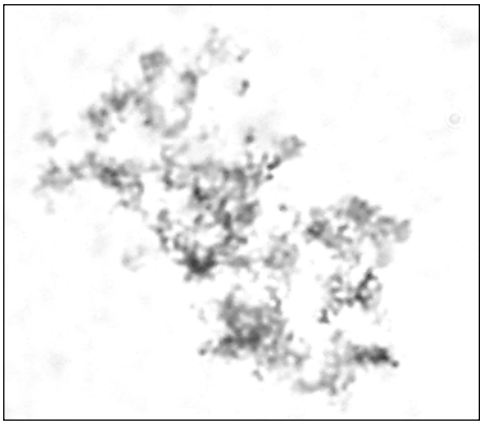


Figure 7. S-phase PCC showing a level potential proliferate of the epithelial differentiated cells in different stage of the cell cycle with fragments of pulverized chromosome.

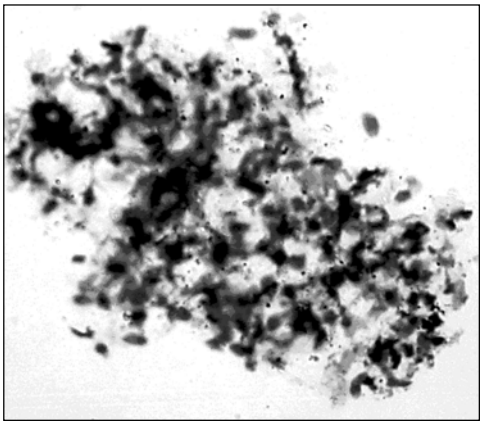


Figure 8. Premature chromosome condensation in skin warts lesions culture: The arrows indicate: interphase cells with two chromatids (G2-PCC) in some chromosomes and others presenting only one chromatid (G1-PCC).

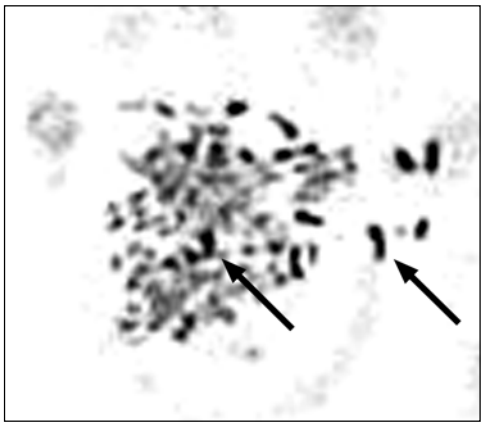


Figure 9. PCC cells in peripheral blood lymphocytes cultures. The arrows indicate: cells with one chromatid per chromosome typical G1-PCC phase and chromosome duplicated (G2-PCC).

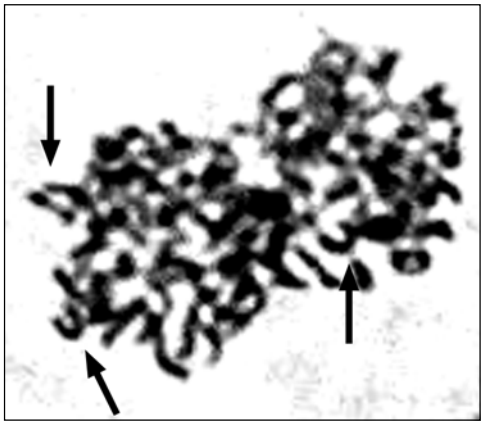
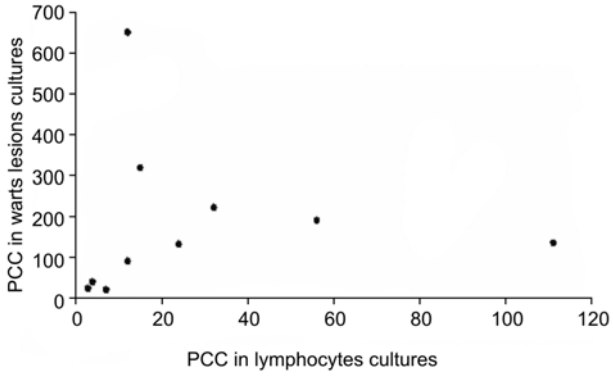


Figure 10. Graphic of dispersion showed no correlation between the two variables of PCC cells in the lymphocytes and PCC cells in the epithelial lesions cultures. Each animal affected clinically by cutaneous papillomatosis was represented by a point in the graphic and both variables are independent themselves.



"prophasing" by Matsui et al., in 1972. PCC can be induced in cycling tissue cells cultures and also in differentiated cells from animal tissues (RAO et al., 1981). Although of the term "telophasing" has not been so well established as the PCC findings, both PCC and telophasing phenomenons are directly relacionated with mitotic and interphasic factors (RAO et al., 1981).

The ability of DNA virus oncoproteins to interfere with mitotic checkpoints has been less studied. Loss of genomic stability is manifested as the increased frequency of cytogenetic aberrations. Control mechanisms regulated by checkpoints induced cell cycle entry into S-phase or mitosis when an event such as DNA damage (THOMPSON et al., 1997). In tumor cell populations, have been commonly observed numerical and structural chromosomal abnormalities (THOMPSON et al., 1997), when the premature condensation of S-phase chromatin results in fragmented configuration. It's occurs because cells in S-phase was interrupted and the phenomenon of chromosome fragmentation or pulverization suggest that the chromosome core is missing or broken (RAO et al., 1981). Our findings are in accordance to Rao et al., 1981.

Basically, the control cellular cycle has been done by regulators proteins which acting in the G1 and G2 stages. Mutations in tumors suppress genes induced the altered proteins which no interrupt cellular division process and no repair damage DNA, so the cells transmitted this fails to the subsequent divisions originating tumors formation. Mutations in these genes are common events in the cancer that many times not occur alone (FILHO & GTTÁS., 2001).

The micronucleus test (MN) are identified in the interphase of cellular division and presenting small cytoplasmatics corpuscles corresponding acentrics fragments resulted of chromosomal deletions or whole chromosomes which late during the anaphasing. This test has been applied in evaluation of humans populations exposit to mutagenic and carcinogenic agents (FILHO & GTTÁS., 2001).

Molecular mechanisms of chromosome condensation are still not completely understood. DNA virus genome used strategies to facilitate their replication and maturation in host cells, using the machinery of cellular replication. Small DNA virus, such as *Simian Virus* 40 (SV40) and *Papillomavirus* (PV), modulate the process of controlling the cell cycle and promote the entry into the S-phase, allowing the DNA polymerase of the host can be used for viral replication (LU et al., 2006 apud LEE et al., 2007). In addition to the PV and SV40, the ability to induce chromosome condensation is stored in other viruses such as herpesvirus, for example, (LEE et al., 2007). *Epstein-Barr Virus* (EBV) classified in the family *Herpesviridae*, *Gammaherpesvirinae*, after primary infection becomes latent and its episomal genome replicate itself during the S-phase of the cell cycle (LEE et al., 2007).

Until the date, morphological changes in the cellular chromatin architecture had been reported by *Sendai Virus* infections (RAO et al., 1981) and EBV replication (ITO et al., 2002; LEE et al. 2007) that able to induce chromosome condensation and premature chromosome had not been yet described in papillomavirus infection. So, this is the first relate of detection of PCC cells present in lymphocytes and epithelial lesions cells cultures infected by *bovine papillomavirus*.

The number of PCC cells was found in larger number mainly in the skin warts lesions cultures causing a possible instability cromossomal and the PCC findings, most of them were showed up at the S stage of cellular cycle. According to studies by Duelli and Lazebnik, (2007), this S-phase refers to multiple breaks of DNA fragments resulting in multiple chromosomal supported by the appearance of pulverized cell. The chromatin of warts lesions cells have to be highly similar with the studies by Lee and coworkers (2007) refers to the chromatin of nasopharyngeal carcinoma cells is highly condensed when positive for the EBV.

Skin warts lesions cultures and peripheral blood lymphocyte cells cultures showed phenotype similar to Prophase according to the study of Trimborn and coworkers (2004) to refer the chromosomes aberrations found in premature genetic mutations.

This cytogenetic study is pioneer in the detection of premature chromosomes condensation (PCC) in this species investigated. The results suggested that the viral genome interaction with the chromatin of host cells was due to the high levels of proliferation of infected cells by the presence of high load viral.

Our results indicate that the PCC phenomenon was induced by the viral infection during the differentiation of the tumors cells and lymphocytes studied. Premature chromosome condensations are identified in differentiated cells from animal tissue and also in bovines lymphocytes infected naturally by papillomavirus. Studies suggest the lymphocytes as the site of viral latent infection and the papillomavirus can be the cause by the induction of chromosomes fragilities by structural and numerical level. We thought that p53 regulates G1 checkpoints, was mutated in as blood cells as tumors cells, so in addition, S-phase cells resulting premature chromosomes showed evidence of genomic instability.

We supposed that has been occurred an expression of a mutant tumor suppressor gene relative to viral oncoproteins E6 and E7 generating increases in sensitivity to biologically induced S-phase PCC and mitotic disruption in the cellular cycle. So we suggested that PV oncoproteins and viral activity deregulated mitotic checkpoints interfering cyclin-dependent Kinase (cdk) complexes and propitiated their potential in tumor virus-mediated oncogenesis.

Such fact denotes the importance of the cytogenetic

analysis in the papillomatosis study in order to contribute for the knowledge of the chromosome abnormalities in lymphocytes cultures and tumors cells cultures to identify the types of chromosomes rearrangements. The next step of our research is using banding cytogenetic to shoe specific chromosome bands, karyotyping analysis and probably to apply molecular cytogenetic technique with fluorescence in situ hybridization (FISH) to show an increase significantly in the chromosomal aberrations detection included those anomalies not observed in conventional cytogenetic and explained the markers anomalies in specific chromosomes.

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