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BIOLOGY

Alternatives for obtaining a continuous cell line from *Apis mellifera*

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ABSTRACT: *In worldwide there are reports of a significant decrease in colonies of the species Apis mellifera, caused by several factors, including viral infections. In order to study and diagnose illnesses caused by viruses, in vitro cell culture is used as a valuable tool. Yet, there are still no immortalized cell lines of honey bee Apis mellifera. Primary cell cultures are promising for this purpose and can supply the lack of continuous strains, but their establishment is difficult and laborious, which often makes them unfeasible for many research centers. Through the use of cell immortalization techniques, it is possible to develop continuous cell lines and thus benefit, in different ways, research related to different species of bees. The choice of technique is challenging, since in addition to the ability to remain viable for countless passages, cells must keep the genotype and phenotype similar or identical to the original tissue. This review intends to present methodologies that can be used to immortalize Apis mellifera cells, aiming to establish a cell line. The genotypic and phenotypic implications of each technique are evaluated, and the purpose of the cell line to be developed.*
Key words: *immortalization, cell culture, cell line, honeybee.*

Alternativas para a obtenção de uma linhagem celular contínua de abelhas *Apis mellifera*

RESUMO: *Ao redor do mundo há relatos da diminuição significativa de colônias da espécie Apis mellifera, causada por diversos fatores, incluindo infecções virais. Para estudo e diagnóstico de enfermidades causadas por vírus utiliza-se, como uma ferramenta valiosa, o cultivo celular in vitro. Contudo, ainda não existem linhagens celulares imortalizadas de abelhas Apis mellifera. Os cultivos celulares primários são promissores para este fim e podem suprir a falta de linhagens contínuas, porém seu estabelecimento é difícil e laborioso o que, muitas vezes, os torna inviáveis para muitos centros de pesquisa. Através do uso de técnicas de imortalização celular é possível desenvolver linhagens contínuas de células e assim beneficiar, de diversas formas, as pesquisas relacionadas às diferentes espécies de abelhas. A escolha da técnica é desafiadora, visto que, além da capacidade de permanecer viável por inúmeras passagens, as células devem manter o genótipo e fenótipo semelhante ou idêntico ao tecido original. O objetivo deste trabalho é apresentar metodologias que podem ser utilizadas para imortalização de células de Apis mellifera, visando o estabelecimento de uma linhagem celular. São avaliadas as implicações genotípicas e fenotípicas de cada técnica, e a finalidade da linhagem celular a ser desenvolvida.*
Palavras-chave: *imortalização, cultivos celulares, linhagem celular, abelhas.*

INTRODUCTION

The combination of factors, such as environmental stress and infections by various pathogens, are causing a drastic reduction in bee populations across the planet (PIRES et al., 2016). Viral infections pose a significant threat to beekeeping (GISDER & GENERSCH, 2017). Viruses can persist naturally in colonies, using a variety of transmission and replication pathways, and often may not cause visible symptoms. However, the presence of these microorganisms, associated with the accumulation of environmental factors, can trigger the death of an entire colony (VANENGELSDORP et al., 2017). An increasing number of viruses have been identified in *Apis mellifera*, many of them being associated with the disappearance of these bees (BEAUREPAIRE et al., 2020).

Viruses require the help of cellular machinery to replicate and produce new particles (VIRGIN et al., 2009), which makes cell lines a valuable tool for studying the interaction of these

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microorganisms with cells, gene expression, DNA and RNA replication, among others (GENERSCH et al., 2013). A cell line can be obtained from primary cultures, which generally give rise to finite lines, with few passages and low proliferation. However, neoplastic or immortalized tissues can give rise to continuous cell lines, with a high rate of cell proliferation and an indefinite number of passages (CASTILHO et al., 2008). Yet, the lack of immortalized cell cultures of *Apis mellifera* has been a limiting factor for research in this species (CARRILLO-TRIP et al., 2016) This review aims to discuss viable alternatives for obtaining a continuous cell line of *Apis mellifera* bees, through techniques of cell immortalization.

Cell culture

Since the development of the first invertebrate cell lines (GRACE, 1962), more than 1100 insect cell lines have already been registered, according to the Cellossaurus® database (ExPASy - Swiss Institute of Bioinformatics -SIB, 2020). Among them, only two of *Apis mellifera*, AmE-711 (GOBLIRSCH et al., 2013) and MYN9 (KITAGISHI et al., 2011), considering all insect species, are described. The AmE-711 is originated from honeybee eggs and is a strain that has not undergone an immortalization process, and presents characteristics of a finite lineage, such as being ~~, is~~ limited to a few passages, low cell proliferation and is the one which the scientific society does not have commercial access. This cell line proved difficult to maintain and crashed in 2015 but have been recovered and adapted to a commercially medium (CARRILLO-TRIPP et al., 2016; BEAUREPAIRE et al., 2020; GUO et al., 2020). The MYN9 is originated from honeybee larvae and is the only report of cultivation of this species that was induced to immortalization. The immortalization was performed using the introduction of human c-myc proto-oncogene by conventional lipofection technique, and subculture at eight months until the end of project (KITAGISHI et al., 2011). However, this strain is not available in any cell bank, and there are no reports of its use.

As a way to overcome this situation of the unavailability of *Apis mellifera* cell lines, studies with viruses, currently carried out, use primary or subcultivated cultures from different tissues and at different stages of development of this species (GENERSCH et al., 2013; BEAUREPAIRE et al., 2020). So far, several primary cultures with 6 to 135 days have been reported, originating from eggs, pupae, intestines, nervous tissue, among others (POPPINGA et al., 2012; JU & GHIL, 2015; GUO et al., 2020).

The Laboratory of Virology and Immunology, at the Veterinary Faculty of Universidade Federal de Pelotas - UFPel - has been developing studies for the stabilization of primary cultures and the immortalization of a cell line of *Apis mellifera*. So far, our research group has accumulated promising results, with the development of more than 60 primary cultures originating from eggs and bee larvae, which have remained viable for more than 4 months and with several passages. The main problems observed, such as contamination, culture media and cultivation patterns, have already been overcome. Our group also successfully managed primary cultures maintaining a high rate of proliferation even after eight months of freezing. This fact is extremely relevant, as it allows continuous work in the laboratory even in winter, when there is no laying by the queen, which would make it impossible to make primary cultures from eggs. The next step is the elaboration of a continuous lineage of this species, through cellular immortalization.

Alternatives to cell immortalization

In general, cell immortalization occurs when the cell loses the pathways of verification of the cell cycle, responsible for senescence and apoptosis (WRIGHT & SHAY, 1992; MAQSOOD et al., 2013). Among some genes related to these processes, *p53, p16* and *pRb* stand out (BARNES et al., 2019). The establishment of a cell line can be achieved through the adoption of several immortalization techniques or protocols, including the use of radiation, carcinogenic agents, chemicals, viruses and recombinant DNA vectors that express oncogenes (STACEY, 2006). Cell immortalization is seen as a challenge for scientific communities and, in the case of insect cells, this challenge becomes even greater due to the lack of genetic methods for derivation of new cell lines (LI et al., 2012; MAQSOOD et al., 2013). Some examples of immortalized cell lines are shown in table 1.

myc Proto-oncogenes

The *myc* gene group has great importance in the cell cycle, as the increase in its expression directly affects the transcription of genes responsible for cell proliferation, transformation, differentiation, metabolism, genomic maintenance and adhesion, but also apoptosis (DANG et al., 2006; GARCÍA-GUTIÉRREZ et al., 2019). The Induction of an increase in intracellular *myc* levels develop a mutagenic effect and also cellular stress. The mutagenic and cell stress environment enhance the rate in intracellular

Table 1 - Agents of immortalization, targets, and cell lines.

Immortalization agent	Target/action	cell line	reference
myc proto-oncogene	Increase expression of cell cycle genes	MYN9 (*Apis mellifera*)	DANG et al., 2006; KITAGISHI et al., 2011
ras proto-oncogene	Over-stimulation of signaling pathways	Ras[V12]-H1 (*Drosophila melanogaster*); Ras[V12]-H7 (*Drosophila melanogaster*).	DEQUÉANT et al., 2015; SIMCOX et., 2008
htert	Reactivation of telomerase	hTERT-BTY (*Bos taurus*); hTERT-AEC II (*Bos taurus*).	SU et al., 2013; MAO et al., 2018
mnng	Several damage methylations to base pairs of DNA	IOZCAS-Osfu-1, IOZCAS-Osfu-2 (*Ostrinia furnacalis*); IOZCAS-Spex 12 (*Spodoptera exígua*)	GICHNER & VELEMÍNSKÝ, 1982; LI et al., 2012; ZHANG et al., 2014
crispr/cas9	Deletion of CDKN2A	hPrEC-T-deltaN2A (*Homo sapiens*)	ZHAO et al., 2020

oxygen free radical and can promote the p53 protein phosphorylation, which is known as the main gene responsible of cellular apoptosis and senescence (VAFA et al., 2002; WOLPAW & DANG, 2018). The set of changes such as inhibition of apoptosis and cell transformation can promote cell proliferation even in non-nutritious culture media and in the absence of growth factors (EILERS & EISENMAN, 2008).

The importance of *myc* oncogenes in malignancy and maintenance of transformed cells is clearly reported in the literature (KABILOVA et al., 2006; PINTO et al., 2019). Inhibition of *c-myc* expression through *siRNA* in squamous cell carcinoma (SK-N-MC) and neuroblastoma (KB-3-1) cell lines led to loss of cell proliferation (KABILOVA et al., 2006). Mebendazole, which showed anti-cancer activity, was used in gastric cancer cell lines (AGP01), and induced apoptosis due to inhibiting the expression of c-myc (PINTO et al., 2019).

The only transformation of bee cells *in vitro*, described in the literature, was performed by Kitagish et al. (2011) with the insertion of six human proto-oncogenes in bee cells, using the conventional lipofection technique. Among them, *pcDNA3 c-myc* resulted in a cell line, named MYN9, with more than 100 passages. However, there is no other report of this lineage in the literature or deposit in a cell bank. It is believed that the use of *c-myc* genes such *as pcDNA3 c-myc* presents itself as a viable option for the development of a cell line, authenticated and available to other researchers.

Ras proto-oncogene

Ras proteins belong to the superfamily of Ras genes (WENNERBERG et al., 2005). These proteins activate signaling networks that control cell proliferation, growth, and survival (WHITE et al., 1995; SIMANSHU et al., 2017). *Ras* mutations make their proteins constantly expressed, which is widely seen in neoplasms (PRIOR et al., 2012). About 19% of neoplasms in humans have some mutant isoform of the *Ras* genes continuously expressed (PRIOR et al., 2020). This continuous activation of *Ras* results in over-stimulation of it signaling pathways and drives the growth and survival of neoplastic cells (HOLDERFIELD, 2018).

The activation of the *Ras* gene was able to cause an increase in cell proliferation of *Drosophila sp* (KARIM & RUBIN, 1998; BRUMBY & RICHARDSON, 2003). The mutant Ras^{V12} gene had its efficiency proven in the immortalization of Drosophila cells, reducing the time for the confluence of the cells and allowing the cells to be cultured by several passages. Using this oncogene, several Drosophila cell lines were obtained, proving to be a powerful tool for the transformation of primary cultures. The expression of the Ras^{V12} gene promoted cell proliferation by increasing the mitogen-activated protein kinases (MAPK) and by insulin signaling. The control group took 16 to 29 weeks to make the cell confluent, while the introduction of this gene made the cells confluent in three weeks, and allowed for more than 90 duplications, suggesting that the cells became immortal, thus continuous strains (SIMCOX et al., 2008).

Cell lines immortalized by Ras^{V12} activation have increased expression of genes that promote progression in cell cycle and division. The use of this tool in primary cultures of Drosophila embryos, made it possible to obtain cell lines that actively expressed Ras^{V12} (DEQUÉANT et al., 2015). With its proven efficiency in embryonic Drosophila cells, Ras^{V12} can be an important tool in immortalizing our primary cultures of *Apis mellifera*.

Telomerase

Telomerases are ribonucleoproteins that have an RNA component (hTERC) and a catalytic component (hTERT) (HARRINGTON et al., 1997), whose function is to restore base pairs of DNAs lost from telomeres during cell division (MASON et al., 2011). The length of the chromosomes is restored after each cell division in cells that have active telomerases (ZVEREVA et al., 2010). However, this feature is limited to embryonic cells and pluripotent cells. In somatic cells hTERC continues to be expressed, but the expression of the hTERT catalytic component is substantially reduced, losing its function (FORSYTH et al., 2002). With the progressive shortening of the telomeres, the cell reaches its limit of replication and enters senescence (SHAY & WRIGHT, 2000). The introduction of hTERT to the genome of a cell appears to result in direct immortalization, since the cell loses its senescence characteristic, thus obtaining the ability to replicate itself indefinitely (SHAY & WRIGHT, 2005).

The hTERT-BTY cell line was developed from bovine thyroid tissue, using the *hTERT* gene (MAO et al., 2018). These researchers reported more than 100 passages with the cells maintaining their genotypic and phenotypic characteristics. The insertion of the human *hTERT* gene allowed the development of the HTERT-AEC II cell line from the bovine alveolar epithelium (SU et al., 2013). In addition, there are reports of preliminary studies on the activity of telomerases from a cell line of cockroach tissues (ZHANG et al., 2018). These researchers concluded that cells that stabilized *in vitro* culture had greater telomeric activity, being cultured for three years. In addition to the insertion of genes, the expression of the *hTERT* gene can be stimulated by chemical carcinogens, through the exposure of N-methyl-N-nitro-N-nitrosoguanidine (MNNG) (CHENG et al., 2015).

These studies demonstrate the great importance of telomerases in some insect species, substantiating the possibility of using this agent for cellular immortalization of *Apis mellifera*.

N-methyl-N-nitro-N-nitrosoguanidine (MNNG)

MNNG is a monofunctional alkylating agent that causes damage through methylation to base pairs of DNAs, has mutagenic capacity and has been used since 1960 (GICHNER & VELEMÍNSKÝ, 1982). Its action on cell transformation is related to several genetic and epigenetic changes (ZHANG et al., 2009). Its carcinogenic effect has been used as a cell-transforming agent in different species for some decades, both *in vivo* and *in vitro* (OGURA & FUJIWARA, 1987; NARESSE et al., 2009). The treatment with MNNG *in vitro* in different human strains and *in vivo* in rats, demonstrated direct activation of *Ras* oncogenes, a genetic alteration of great importance in carcinogenesis and cell transformation (KANEKO et al., 2002; LEE et al., 2007). Treatment in human intestinal mucosal epithelial cells caused demethylation of hTERT reverse transcriptase precursor genes. These mutations can impact malignancy or cell transformation (CHENG et al., 2015).

The use of this agent is not limited to vertebrate cells. Currently, cell lines of insects immortalized with MNNG are already reported. From primary ovarian cultures of pupae of the species *Ostrinia furnacalis*, two cell lines were developed, called IOZCAS-Osfu-1 and IOZCAS-Osfu-2. After treatment with MNNG, both strains were cultured for 30 passages, successfully cryopreserved and thawed (ZHANG et al., 2014). Another cell line was developed from primary ovary cultures of pupae of the species *Spodoptera exígua*, which, after treatment with MNNG, showed an increase in cell proliferation and longevity. After characterization, this cell line was called IOZCAS-Spex 12 (LI et al., 2012). Thus, MNNG demonstrates its capacity for cell transformation, both in mammalian cells and in insect cells. The use for immortalization of primary cultures of *Apis mellifera* may be just a matter of standardization of the reagents.

CRISPR / Cas9

The Clustered Regularly Interspaced Short Palindromic Repeats - CRISPR / Cas9 was initially discovered as a mechanism of the adaptive immune system of bacteria and *Archaes* (JANSEN et al., 2002). It is a tool for gene modification that allows targeted deletions, insertions and precise changes in the genome of a large number of organisms and cell types (SANDER & JOUNG, 2014). Its use has stood out due to its high specificity, easy handling in vitro and the possibility of simultaneous editing of multiple targets (LINO et al., 2018).

The deletion of the *p53* gene in a canine cell culture made it possible to obtain several populations with infinite useful life, resistance to genotoxicity and absence of carcinogenic characteristics (EUN et al., 2019). The inactivation of two loci of *CDKN2A*, responsible for the expression of *p16INK4A* and *p14ARF*, precursors of the activation of tumor suppressors *p53* and *pRB*, associated with ectopic expression of *hTERT*, was able to immortalize human prostate epithelial cells without any phenotypic changes (ZHAO et al., 2020). By not directly inactivating the tumor suppressors *p53* and *pRB*, it is expected that other properties of the cells remain normal, such as the characteristic cell phenotype, protein production, and genomic stability (MCKINLEY & CHEESEMAN, 2017; ZHAO et al., 2020).

The genome of Africanized bees *Apis mellifera* has been fully sequenced, enabling biomolecular studies of this species (ELSIK et al., 2014; KADRI et al., 2016). Then, the efficiency of the CRISPR / Cas9 technique was proven in this species of bee, since *Mrjp1* (gene of the main protein of royal jelly) was edited and inserted in eggs that generated six genetically modified queens. These queens subsequently oviposited, giving rise to 20 drones, also genetically modified (KOHNO et al., 2016). The editing of genes such as *Pax6*, a transcriptional regulatory gene in the development of the nervous and ocular system, and Mrjp1 in *Apis mellifera* with the use of CRISPR / Cas9 demonstrated high efficiency, with editing rates of up to 100% of the samples used (HU et al., 2019). The efficiency of this technique in editing *Apis mellifera* genes opens the way for the modification of genes of interest, for cellular immortalization.

With the use of CRISPR / Cas9 to immortalize mammalian cell cultures and demonstrated the efficiency in gene editing in the species of interest, this technique can be of great value in the process of immortalizing bee cells, through the deletion of precursor genes senescence.

CONCLUSION

Many cell immortalization techniques are currently described for mammals, but they need to be adapted for use in bee cells. The choice of technique and immortalizing agent is fundamental and challenging, because besides the ability to remain viable for countless passages, cells must maintain the genotype and phenotype similar or identical to the original tissue. In this review, some agents for the immortalization of primary cultures were presented, which may be useful in the development of a continuous cell line from *Apis mellifera* bee cells. However, some techniques or targets of gene editing can generate undesirable changes, which highlights the need for an individual assessment. This assessment should consider the specific agent and the purpose for which the cell line will be used.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final.

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