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EFFECT OF METFORMIN ON ABCB1 AND AMPK GENE EXPRESSION INADOLESCENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA.

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Effect of metformin on ABCB1 and AMPK gene expression in adolescents with acute lymphoblastic leukemia.

BACKGROUND

Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is a malignant neoplasm that originates from lymphoid progenitors in the bone marrow. In most countries it is the most common malignancy in children, and is the most frequent cause of death secondary to cancer in children under 20 years of age. [1,2,3] In the United States, the incidence of ALL varies according to ethnicity, with Hispanics being the most affected, with figures up to 40.9 cases per million. [4] In Mexico, according to the records of the popular insurance program, leukemia has a prevalence of 49.8%, in the period 2007-2012, 83% of these correspond to ALL.[5]

Clinical characteristic at diagnosis of ALL include anemic, hemorrhagic and infiltrative syndromes. [3] ALL in children has had progressive improvement due to multimodal therapy and risk stratification, offering the patient an intensified treatment depending on the risk of relapse. This risk stratification is based on the patient's clinical data, biological characteristics of the leukemic cells and early response to treatment, which predict the likelihood of relapse.[3] These help to select the intensity of treatment, to achieve the best chance of cure. Patient age and leukocyte count at diagnosis are among the most important, based on this, age less than 1 year and greater than 10 years confers an adverse prognosis. [6,7] Genetic alterations have also been related to the prognosis of children with ALL. [8-12]

Early response to treatment refers to the time required to eliminate the bulk of the leukemic cell population to undetectable levels; this is the single most powerful prognostic factor in pediatric ALL. Measurement of submicroscopic levels of minimal residual disease (1 leukemic cell in 10^{4-5} normal cells) can be obtained by polymerase chain reaction amplification or flow cytometry. [13,14] The risk of treatment failure and death is 3 to 5 times higher in patients with minimal residual disease levels of 0.01% or greaterat the end of induction than those with levels less than 0.01%. [15]

Treatment of ALL

The combination of chemotherapy agents achieves remission in 80-90% of children with ALL, especially with intensive protocols of 6-8 drugs in 4-8 weeks in the remission induction and consolidation phases, achieving in our days rates of up to 85% event-free survival and 90% overall survival, in developed countries.[3]

Current regimens include induction therapy consisting of 4-6 weeks and include steroid, vincristine, asparaginase, anthracyclines and intrathecal therapy. Consolidation includes 6 months of a combination of

intense chemotherapy that is designed to consolidate remission and prevent the development of leukemia in the central nervous system. This phase includes high-dose methotrexate. And finally, patients receive maintenance treatment with low-intensity chemotherapy for 18 to 30 months. [3] Another important part of the treatment is therapy directed to the central nervous system. [16, 17]

Treatment failure occurs in 15-20% of children with ALL in developed countries, leading to lower cure rates after relapse. When relapse occurs during treatment, the probability of achieving a second remission is 50-70% and only 20-30% of patients are cured. [18,19]

ALL in adolescents

The treatment of adolescents with ALL has always been challenging, as the mere fact of being older than 10 years carries an adverse prognosis. These patients diagnosed with ALL have shown very little prognostic benefit compared to younger patients with ALL younger than 10 years. It has even been reported that adolescents and young adults have 7.7 to 10.5 times higher risk of relapse compared to children between 1 and 10 years of age. [20] The factors that cause this age group to have worse survival rates are unknown. Some proposals are heterogeneity in the biology of leukemia, host factors, both physiological and psychosocial, and the therapeutic approach proposed by the health care team. [21-23]

Multidrug resistance (MDR)

Despite advances in treatment strategies, approximately 20% of patients will experience relapse. One of the main theories of relapse is the presence of multidrug resistance. Several mechanisms responsible for drug resistance have been described, both in vivo and in vitro. One of the most important mechanisms is the increased expression of ATP-binding cassette transporters or ABC transporters (ATP-binding cassette transporters). This is a superfamily of 49 human ABC genes, which are classified into 8 subfamilies from ABC-A to ABC-G and ANSA, based on the degree of homology of their sequences. [24] These genes are specialized in energy-dependent cellular transport and are involved in a wide range of events, such as removal of harmful substances, secretion of toxins, mobilization of ions and peptides, as well as cell signaling. [25]

Within this family, ABCB1/MDR1, ABCC1/MRP1, ABCC3/MRP3 and ABCG2/BCRP genes have been evaluated in several studies, with controversial results in drug resistance in pediatric ALL. [26-30] Inclusively, Rahgozar, et al. demonstrated an increased risk of obtaining positive minimal residual disease in expression of ABCA2, ABCA3, MDR1 and MRP1 genes. [31,32,33]

Metformin and cancer

Metformin is an antidiabetic drug used in patients with type 2 diabetes mellitus. Metformin is a synthetic biguanide, whose main mechanism of action is through mitochondrial activity, among others that are not well described. [34] It has been experimentally demonstrated that high levels of insulin and glucose promote cell growth in vitro, proposing a possible mechanism of action of metformin.

mechanisms directly involving cancer and DM2, via hyperinsulinemia, hyperglycemia and inflammation. [35,36] In some epidemiological cohort studies of patients with type 2 diabetes, in which the use of metformin alone or combined with sulfonylurea, decreased the risk of developing cancer, compared to those exposed to insulin. [37] These findings have been replicated in different reports with consistent results. Currently, there are many articles on the use of metformin in cancer, however, the most solid evidence is in breast, colorectal, ovarian and endometrial cancer in adults; although more evidence is required to evaluate the therapeutic effect in other cancers. [38]

The main antitumor mechanism of antidiabetic drugs such as biguanide and thiazolindiones, which have been shown in various cell lines to inhibit cancer cells, is likely due to their disruption of the AKT/mTOR signaling pathway in various ways, resulting in differences in leukemic cell proliferation, apoptosis and chemo-sensitivity. Some researchers have identified activated adenosine 5'-monophosphate protein kinase, or AMPK, which is an energy regulator for homeostasis, as a target for ALL treatment because of its effect on cell proliferation, cell cycle regulation, and its crosstalk with critical metabolic and oncogenic pathways.[39-40]

Metformin has a positive charge, which accumulates in the mitochondrial matrix and inhibits complex I of the respiratory chain, preventing the oxidation of NADPH with the consequent decrease in ATP levels, which causes the accumulation of AMP. When there is an elevated AMP/ATP ratio, the activity of LBK1 (liver kinase B1 or serine/threonine kinase11 STK11) responsible for the phosphorylation and activation of AMPK, a metabolic sensor that inhibits energy-intensive cellular processes as in neoplastic cells, is promoted.

AMPK is activated due to the change in ATP levels, shifting the cell from an anabolic to a catabolic state, restoring energy balance. [41-43] In addition, activated AMPK phosphorylates TSC2 (tuberous sclerosis complex 2) which stimulates the activity of GTPaseRheb (Ras homolog enriched in brain), with subsequent inactivation of mTOR (mammalian tardet of rapamycin), essential for cell proliferation because it regulates the expression of cyclin D1, HIF-1alpha/2alpha (hypoxia-inducible factors), and the so-called Yamanaka transcription factors, OCT4, KLF4, SOX2, c-Myc.[43-45]

AMPK activation inhibits mTOR, and the transcriptional regulation of FOXO3A program, which is important in autophagic cell death of persistently stressed cells. In summary, treatment of pre-B ALL and T-cell ALL cell lines with AMPK activators, including metformin, has been shown to significantly induce cell growth inhibition and apoptosis.[46,47]

In addition to the systemic effect of metformin on insulin levels, this drug can suppress malignant cells by a direct mechanism involving cancer stem cells, epithelial-mesenchymal transition (EMT) and cellular senescence. [48,49]

Studies, such as that of Jingxuan, demonstrated that the use of metformin improves the chemosensitivity of the anthracyclics, L-asparaginase, vincristine and ethoposide, through the impact on the AKT/mTOR pathway, which are used in LAL.[50] Other mechanisms involving metformin with other metabolic pathways involving the cancer response have also been described. [43,47, 51-55]

Another possible effect of metformin is on the multidrug resistance gene family mentioned above.[25]Although chemotherapy resistant cells is a fundamental problem for oncologists, there are few studies on ABCB1/MDR1. However, metformin has shown a decrease in the expression of MDR1, mainly through two pathways, the first one initiates with the activation of AMPK which decreases the activity of adenylate cyclase which in turn reduces the activity of PKA (protein kinase A), preventing the final phosphorylation of CREB, necessary to carry out the transcriptional function. [56,57] Second, metformin reduces NF-kB levels through inhibition of the TNF-alpha receptor signaling cascade. [57] Some drugs, such as vincristine, anthracyclines, epipodophyllotoxins and tyrosine kinase inhibitors, are substrates of these receptors, which are present in several types of cancer. [25,58]

Metformin in pediatrics

In pediatrics, metformin is a drug approved by the Food and Drug Administration (FDA) for the treatment of type 2 diabetes, and is indicated for children over 10 years of age based on studies that demonstrate its safety and clinical benefit for type 2 diabetes at this age. [59] For this indication, it is generally dosed twice daily with an excellent safety profile in this age range. [60] Pharmacokinetic data in children between 12 and 16 years of age have been shown to be within 5% of those compared to controls for age and gender in adults, with C_{max}898ng/ml and $ABC_{(inf)}$ 6311ng hr/ml. [61] The recommended phase 2 dose for metformin is 1000mg/m2/day, with the main side effects reported at the gastrointestinal and metabolic level. [40]

The only study so far where metformin has been used in patients with ALL was published by Trucco, et.al., where he used a traditional induction scheme with 4 drugs plus metformin in pediatric patients with refractory and relapsed ALL, in 14 patients, without reaching definitive conclusions regarding the response of ALL and metformin, however he demonstrated that the use of metformin is safe with an acceptable toxicity profile.[40]

RESEARCH QUESTION

What is the effect of metformin use on ABCB1/MDR1 and AMPK gene expression in the remission induction phase in adolescent patients with newly diagnosed ALL?

PROBLEM STATEMENT

ALL is the most common oncologic disease in the pediatric population, representing 35% of childhood cancer cases in world statistics, and up to almost 50% according to Mexico`s national statistics. In countries with limited resources, long-term survival is reported to be greater than 90%. In countries such as Mexico, the 5 year overall survival of this disease has been described to be around 50%, and in those patients with poor prognostic factors even lower, including adolescents.

The expression of the ABCB1/MDR1 and AMPK gene in adolescents, as well as in patients with ALL and its variation during treatment, is currently unknown.

In recent years, the use of metformin has proven to be useful in the treatment of different hematological malignancies and solid tumors, such as breast cancer and leukemia, through different metabolic pathways. Although the usefulness of this drug has been demonstrated in adults, in pediatrics it is still an unproven field. Clinical trials in children and adolescents are beginning to be conducted in refractory or relapsed solid tumors; however, there are none in acute lymphoblastic leukemia.

To date, the benefit of metformin use in ALL in pediatrics, especially in the high-risk group of adolescents, and the interaction it may have with ABCB1/MDR1 and AMPK gene expression, has not been reported. Therefore, it is necessary to evaluate the expression of these genes and the usefulness of metformin in adolescents with acute lymphoblastic leukemia.

JUSTIFICATION

The description of ABCB1/MDR1 and AMPK gene expression in adolescents with ALL may help to understand their involvement in the biological behavior of ALL in this population.

The search for new alternatives in cancer treatment to improve patient survival is one of the main objectives of current research. The repositioning of old drugs in cancer treatment is one of the lines being analyzed, as is the case of metformin.

In basic and clinical studies, the antineoplastic activity of metformin has been demonstrated. It has been studied in breast cancer, prostate cancer and hematological neoplasms, all in adults. Therefore, transferring these studies to other populations, such as adolescents, may contribute to improve the survival rate in this group with poor prognosis in ALL.

The generation of knowledge regarding the usefulness of metformin in this specific group of patients can lay the groundwork for research on the effect of this drug in solid tumors and other pediatric neoplasms.

HYPOTHESIS

If metformin is added to standard chemotherapy during the remission induction phase in Mexican adolescents with newly diagnosed ALL, then it will decrease the level of ABCB1/MDR1 gene mRNA expression and increase AMPK expression levels, at the end of this treatment period.

OBJECTIVES

General:

- To evaluate the efficacy of metformin in modifying ABCB1 and AMPK gene expression in adolescents diagnosed with ALL during the remission induction phase.

Specific

- To describe the expression levels of ABCB1/MDR1 and AMPK genes in adolescents with ALL at diagnosis and in healthy controls.

- To compare the expression levels of ABCB1/MDR1 and AMPK genes at diagnosis and at the end of induction in adolescents with ALL treated with conventional chemotherapy and those additionally treated with metformin.

- To compare overall and disease-free survival in adolescents with ALL treated in relation to ABCB1/MDR1, and AMPK gene expression.

- To compare overall and disease-free survival in adolescents with ALL treated with conventional chemotherapy with those treated with convectional chemotherapy plus metformin.

METHODOLOGY

Type and design of study
Type of study: randomized clinical trial
Study design: analytical, experimental, longitudinal and prospective study.

Population
The population to be studied are patients with newly diagnosed ALL between 10 and 21 years.

Eligible population: patients with newly diagnosed ALL between 10 and 21 years old, diagnosed at the Hospital General de México “Dr. Eduardo Liceaga” from February 2021 to December 2023.

Inclusion, exclusion and elimination criteria

- Inclusion criteria:
  1. Patients aged between 10 and 21 years with a new diagnosis of ALL at the Hospital General de México, Dr Eduardo Licega.
  2. Patients and legally authorized person who sign the informed consent.

- Exclusion criteria:
  1. Patients with antineoplastic treatment prior to admission.
  2. Patients with Down syndrome.

- Elimination criteria:
  1. Patients who abandon treatment for more than a few weeks.4
  2. Patients who withdraw informed consent

Sample size

To calculate the sample size, we used data from the publication by Ramos Peñafiel[63], where a decrease in the expression pattern of ABCB1 gene expression from high to low expression after metformin administration was 50%.

The sample size calculation was based on the formula for estimating a proportion, with a confidence level of 95% and a precision of 5%:

$$n = \frac{Z_{\alpha}^{2} * p * q}{d^{2}}$$

A sample number of 8 patients in each group is obtained, assuming a loss of 20%, 10 patients are calculated for each group.

Definition of the variables to be evaluated and how they are to be measured

Variable	Conceptual Definition	Unit of measurement	Type of variable	Coding
Age	Time elapsed in years since birth.	Years	Quantitative	Not applicable

Gender	Male or female phenotype of the person.	Male/female	Qualitative Dichotomous	0: male 1: female
Leukocyte count at diagnosis	Number of total leukocytes reported on complete blood count at diagnosis.	X10/ml^3	Quantitative	Not applicable
Hemoglobin at diagnosis	Measurement of the amount of oxygen transport proteinreported in initial complete blood count.	g/dL	Quantitative	Not applicable
Platelet count at diagnosis	Measurement of the number of platelet fragments produced by the megakaryocytes, reported in the Initial complete blood count.	X10/ml^3	Quantitative	Not applicable
Immunophenotype of leukemia lymphoblastic	Cellular variant of acute lymphoblastic leukemia	B/ T	Qualitative dichotomous	0: strain B 1: lineage T
Cytogenetic alterations	Alterations in the genetic structure of leukemic cells.	None/ t(9;22)/ t(12,21)/ t(4,11), other	Qualitative polytomous	0: none 1: t(9;22), 2: t(12,21) , 3: t(4,11), 4: other
MDR-1 and AMPK gene expression.	Amplification of gene expression by RT-PCR	No expression / lowexpression / high expression expression	Qualitative polytomous	0: not expressed 1: high expression 2 low expression
Pre-phase response with prednisone	Decrease in total peripheral blood blast count less than 1000 after 7 days of steroid (day 0)	Present/absent	Qualitative dichotomous	0: Yes 1: No
Morphological status at day +14	Presence of blasts in bone marrow sample per day +14 referral induction	M1, M2, M3	Qualitative polytomous	0: M1 1: M2 2: M3
Morphological response at th eend of induction	Presence of blasts in the bone marrow aspirate at the completion of remission induction phase.	M1, M2, M3	Qualitative polytomus	0: M1 1: M2 2: M3
Minimal residual disease at the end of induction.	Flow cytometry measurement of the presence of 1x10-3 blasts.	Positive /Negative	Qualitative dichotomous	0: positive 1: negative
Relapse	Presence of blasts in bone marrow after remission has been achieved.	Present/absent	Qualitative dichotomous	0: absent 1: present

Time to relapse	Time from diagnosis of leukemia to relapse	Days	Quantitative	Not applicable
Death	Cessation of life	Present/absent	Qualitative dichotomous	0: absent 1: present
Time to death	Time from leukemia diagnosis to death	Days	Quantitative	Not applicable

Table 1. Variable definitions.

PROCEDURES

Patients with a newly morphological diagnosis of ALL at the Hospital General de México "Dr. Eduardo Liceaga" will be invited to participate in this trial, and informed consent will be requested from parents and participants.

The data collection is obtained from the patients chart such as age, gender, leukocyte count, hemoglobin, platelet count and immunophenotype.

Peripheral blood samples will be collected in EDTA tube. The sample will be centrifuged obtaining mononuclear cells, and the analysis of ABCB1 and AMPK expression will be performed. This will be done on samples obtained before the start of treatment and at the end of the induction phase. Samples will be collected by peripheral vein puncture, and mononuclear cells will be separated using density gradient. Mononuclear cells are separated and suspended in PBS medium and stored at -70ºC. RNA isolation is performed using TRIzol® (Invitrogen/life Technologies), and will be stored at -80ºC until use. For cDNA synthesis, 2g of RNA, final volume of 20L will be combined with 200U of MMLV RT enzyme (Invitrogen, Carlsbad, CA, USA).

Expression of mRNA levels will be measured using the TaqMan gene expression assay (Applied Biosystems Foster city, CA, USA). The cut-off point between low and high expression will be determined according to mean values obtained in healthy controls, selected during case recruitment in a 2:1 ratio for each case.

Regarding treatment, patients will be randomized into 2 groups, where in both cases the conventional remission induction treatment will be given, and to intervention group metformin will be added at a dose of 1000mgm2Scday, divided into two doses, with a maximum dose of 850mg every 8 hour,orally.

The assignment will be made by means of the block randomization technique, in which, from a table of random digits, two-digit numbers are selected, each of which corresponds to a block of 6 patients, to which the intervention is assigned in the corresponding sequence.

All patients will start treatment with steroid window, with prednisone at a dose of 60mgm2Scday (maximum dose 100mg/d), for 7 days (from day -7 to-1). Patients in the study group (treatment with conventional chemotherapy + metformin) are added metformin hydrochloride orally, at a dose of 1000mgm2Scday, divided into two doses, with a maximum dose of 850mg every 8 h starting from day -7 of treatment. On day 0 the response to the pre-phase with prednisone (60mgm2scd) will be assessed by means of blood biometry with differential count and blast count.

Both groups will start remission induction and consolidation treatment according to the Institutional High Risk protocol, which includes administration of vincristine, prednisone, daunorubicin and L-asparaginase. Patients assigned to intervention group will also continue with metformin at the established doses.

A bone marrow exam will be performed at day +14 of remission induction to determine the marrow response to chemotherapy. A bone marrow aspirate will be performed at the end of remission induction to evaluate the response to this phase of treatment with morphological study and minimal residual disease, as well as determination of the expression of the ABCB1/MDR1 and AMPK gene with the methodology described above. At the end of remission induction, metformin will be discontinued in group assigned to it.

Patients will be followed up for 12 months from the start of treatment, and any relapses of the disease or deaths, as well as the time at which they were diagnosed, will be recorded on the data collection sheet.

STATISTICAL ANALYSIS

Data analysis will be carried out using the Statistical Package for Social Sciences (SPSS) version 25.0. Descriptive data will be expressed in percentages and absolute values for qualitative variables, and will be described in medians, ranges, means and standard deviation for quantitative variables.

The Kolmogorov-Smirnov test will be performed for continuous variables to determine if they have normal distribution; if they have normal distribution, both groups will be analyzed by means of the t-student test; if they do not have normal distribution, the chi-square test, Fisher's exact test and Mann Whitney U test will be used for the bivariate analysis. To compare the expression of ABCB1/MDR1 and AMPK genes at the time of diagnosis and at the end of induction, McNemar's test will be used.

For the calculation of the overall and disease-free survival function, the Kaplan Meier survival test will be used for both groups and the differences between groups will be analyzed by means of the log Rank test, both for overall and disease-free survival. Similarly, the overall and disease-free survival function will be analyzed with the Kaplan Meier test and log rank for ABCB1/MDR1 and AMPK gene expression.

ETHICAL AND BIOSAFETY ASPECTS

According to the General Health Law regarding research on human subjects, this protocol is considered a higher than minimal risk research, since randomized methods of assignment to therapeutic schemes are used.

This protocol takes into account the Mexican General Health Law regarding the provision of health care services and health research, as well as national and international ethical standards.

This study will be carried out with strict observance of recognized scientific principles and respect and confidential handling of the data obtained. The security mechanisms to maintain the ethical aspects of this study are:

1. Review of this protocol by the research and ethics committees of the Hospital General de México "Dr. Eduardo Liceaga".
2. All information obtained in this study will be made available to the Ethics and Research Committees.
3. The confidentiality of the study information is assured, as well as the identity of the patients. The identification data of the participants will be codified, assigning a consecutive participation number. The identification information and the relation between the identification numbers and the data obtained will be known only to the participating investigators and will be preserved confidentially in electronic media.
4. There will be no charge for the study, nor will there be any consistency for participation in this study. Refusal to participate in the study will not influence the patient's treatment or clinical care.

5. Informed consent and assent will be requested for participation in this study, respecting the autonomy of the participants.
6. The principles of fairness are respected since the participants will be randomly assigned by blocks.

The responsible investigators have no conflicts of interest in the conduct of this study or its publication.

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