Differential Response of Human Amniotic Membrane Proteins in Hepatocellular Carcinoma: Analysis of the Glycolytic Metabolism

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Research Article

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Abstract

Background: The human Amniotic Membrane (hAM) has been studied as a potential therapeutic option in cancer, namely in hepatocellular carcinoma. Previously, our research group evaluated the effect of human Amniotic Membrane Protein Extracts (hAMPE) in cancer therapy, demonstrating that hAMPE inhibit the metabolic activity of human hepatocellular carcinoma cell lines: Hep3B2.1-7, HepG2 and Huh7.

Therefore, the aim of this study was to evaluate the effect of hAMPE treatment in glucose metabolism of hepatocellular carcinoma.

Methods and Results: Glucose uptake and lactate production was assessed by $^1\text{H-NMR}$, and the expression of several mediators of the glycolytic pathway was evaluated by Western blot or fluorescence. Our results showed that hAMPE treatment increased glucose consumption on Hep3B2.1-7, HepG2, and Huh7 through the increase of GLUT1 in Hep3B2.1-7 and Huh7, and GLUT3 in HepG2 cells. It was observed increased expression of 6-phosphofructokinase (PFK-1L) in all cell lines, indicating that glucose can be converted to pyruvate. Also, it was verified that glucose seems not to be converted to lactate on HepG2 and Huh7 cells, suggesting that hAMPE treatment may contradict the Warburg effect observed in carcinogenesis. In Hep3B2.1-7, the hAMPE treatment induced an increase in expression of lactate dehydrogenase (LDH) and monocarboxylate transporter isoform 4 (MCT4).

Conclusions: Overall, this work highlighted the potential usefulness of hAMPE as anticancer therapy through the modulation of the glycolytic metabolism in human hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) is the most common epithelial tumor and accounts for about 80% of the primary liver tumors [1, 2], which represents a worldwide health problem due to a high morbidity and mortality [3, 4]. Globally, this type of cancer is most frequent in males than in females, and it is most common in less developed countries [5].

The hepatocarcinogenesis is a very complex biological process, in which external stimuli induce genetic changes in hepatocytes leading to uncontrolled proliferation and resistance to cell death [6]. In addition to genetic mutations in HCC, the most metabolic change in tumor cells is an increase of glycolysis rate even in the presence of high oxygen levels and a reduced mitochondrial oxidative phosphorylation, a phenomenon firstly reported by Otto Warburg [7, 8]. One of the main mechanisms of aerobic glycolysis is the conversion of glucose to pyruvate, and consequently, to lactate, which may be achieved by upregulation of glucose transporters (GLUTs), hexokinase II (HKII), phosphofructokinase (PFK), lactate dehydrogenase A (LDHA) and lactate-extruding enzyme monocarboxylate transporter 4 (MCT4) [8, 9].

Usually, HCC is diagnosed in advanced stage and presents a poor prognosis and a reduced survival rate, because at early stages is asymptomatic and the patients develop resistance to conventional treatments [4, 10]. Therefore, and taking into account the absence of effective and selective therapeutic strategies for the treatment of HCC, it is particularly important to find new and effective therapies that allow to reduce

the mortality rate of HCC. In the last years, the human Amniotic Membrane (hAM) has become a subject of study due to their attractive properties, which suggest its application in several human diseases [11]. Considering the attractive properties of hAM, such as immunoregulatory, anti-angiogenic and pro-apoptotic activities, in 2008 Seo *et al.* hypothesized that the hAM can be useful in the treatment of cancer [12], and in 2012, it was published a review article focusing on the attractive properties of hAM in cancer treatment [11]. In an innovative way, Mamede *et al.* have evaluated the effect of Proteins Extracted from Amniotic Membrane (hAMPE) in the metabolic activity of human cancer cell lines, demonstrating that the hAMPE can inhibit the metabolic activity of some human cancer cell lines, including HCC cell lines [13]. It was demonstrated that hAMPE may induce selective cytotoxicity and cell death in Hep3B2.1-7, HepG2 and Huh7 cell lines of HCC. The hAMPE decreased the metabolic activity, protein and DNA content in a dose- and time-dependent, and induced cell morphology alterations in all cell lines of HCC. The mechanism associated with cell death is dependent on the cell line [14], and this can be explained by different biological and genetic characteristics between the HCC cell lines. One of the most important differences between the HCC cell lines Huh7, HepG2, and Hep3B2.1-7 is the difference in p53 status [2, 15]. The expression of p53 is null in Hep3B2.1-7 cells, while it is higher in Huh7 cells than in HepG2 cells. In fact, alterations in the expression and/or activation of p53 are frequent in HCC and can regulate mitochondrial oxidative phosphorylation and glycolysis, where the lack of p53 expression leads to an increase of glycolysis and a decreased mitochondrial oxidative phosphorylation [16]. Thus, it is imperative to clarify the mechanisms involved in the effect of hAMPE in order to contribute to the elucidation of hAM role in cancer treatment. Considering that the hAMPE induce death by apoptosis in all HCC cell lines, associated with increased glycolytic metabolism in cancer cells, this work aims to study the effect of hAMPE treatment in the glucose metabolism of HCC (Figure 1).

Materials And Methods

Collection and preparation of the hAM

The hAM was obtained from donor healthy by elective caesarean section after obtaining the respective informed consent, written according to the guidelines of the Ethical Committee of Coimbra Hospital and University Centre (CHUC-70-12, Coimbra, Portugal). The hAM samples were washed with phosphate buffered solution [PBS: 137 mM NaCl (Sigma, S7653), 2.7 mM KCl (Sigma, P9333), 10 mM Na₂HPO₄ (Sigma, S3139), 2 mM KH₂PO₄ (Sigma, P0662), pH = 7.4] supplemented with 2% antibiotic/antimycotic. The hAMPE was prepared as previously described by our research group [13], which was based on the protocol described by Shao *et al.* [17] with some modifications. The protein extraction was carried out using a mechanical procedure without the addition of detergents. Thus, the hAM was mechanically separated from chorion, it was cut into small pieces that were subsequently minced. Then, PBS was added to pieces, placed on ice, and were homogenized using a Potter for 5 minutes in an ultrasonic bath with ice, followed by centrifugation (14000 g for 15 minutes at 4°C). After centrifugation, the pellet was discarded, the supernatant was collected and stored at -80 °C. The supernatant was filtered using a 0.45

μm filter (Whatman, 10,462,100) under sterile conditions, and finally the proteins were quantified using NanoDrop (ND-1000 Spectrophotometer, USA).

Cell culture and experimental design

The Hep3B2.1-7 and HepG2 cell lines were obtained from the American Type Culture Collection (ATCC), and the Huh7 cell line was purchased from the Japanese Collection of Research Bioresources (JCRB). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM - Sigma, D5648), pH 7.4, supplemented with 10% fetal bovine serum (FBS - Sigma, F7524), 1% antibiotic/antimycotic (Sigma, A5955) and 0.25% sodium pyruvate (Gibco, 11360), and kept at 37°C with 5% CO₂. With the purpose to evaluate the glycolic profile in HCC cell lines after treatment with hAMPE, approximately 6×10^6 cells of each line were cultured until 90% confluence. Then, the culture medium was replaced with control or 1 $\mu\text{g}/\mu\text{L}$ hAMPE-containing medium for 24 h. After time of stimulation, the culture medium was collected, and cells were trypsinized and stored at 80°C.

Glucose consumption and lactate production by nuclear magnetic resonance spectroscopy

Culture medium (600 μL) collected in $t = 0$ h and $t = 24$ h was used for nuclear magnetic resonance spectroscopy (¹H NMR) analysis by means of standard methods, as previously described by Rato *et al.* [18]. For each sample, 40 μL of 10 mM sodium fumarate solutions at 99.9% deuterium oxide (D₂O or 2H₂O) (Cambridge Isotope Laboratories, Cambridge, MA, USA) was added, as an internal reference (6.50 ppm) to quantify the metabolites in solution. ¹H NMR spectra of the collected samples were acquired at 14.1 Tesla, 25°C, using a Varian 600 MHz spectrometer (Varian, Palo Alto, CA, USA) equipped with a 3 mm indirect detection probe with z-gradient. Solvent-suppressed ¹H NMR spectra were acquired with 6 kHz sweep width, using 14 s delay for allowing total proton relaxation, 3 s water presaturation, 45° pulse angle, 3.5 s acquisition time, and at least 64 scans. The metabolite spin systems were assigned using the chemical shifts found in the literature and the following metabolites were determined: lactate production (doublet located at 1.33 ppm) and glucose consumption (doublet at 5.22 ppm) at 0 h and 24 h. The data were processed and displayed offline using the curve-fitting routine supplied with the NUTSpro™ NMR spectral analysis program (Acorn, NMR Inc., Fremont, CA, USA). Glucose consumption and lactate production per cell was calculated by measuring the accumulated variation (versus time zero) at the defined time period 24 h and dividing it by the total number of cells in each flask. The concentrations obtained for the different metabolites were corrected for the removed volumes of medium.

Evaluation of GLUT1 and GLUT3 expression by fluorescence

In order to evaluate the glucose transporters expression, approximately 1×10^6 Huh7, HepG2 and Hep3B2.1-7 cells were incubated with 1 $\mu\text{g}/\mu\text{L}$ of hAMPE for 24 h. After this incubation period, cells were trypsinized, centrifuged (1300 x g for 5 min) and washed with PBS. Subsequently, cells were incubated with anti-GLUT1 conjugated with phycoerythrin (PE) fluorochrome (3 μL - R&D Systems, FAB1418P) or anti-GLUT3 (3 μL - R&D Systems, MAB1415) for 15 minutes in the dark. Next, the cells were washed with

PBS by centrifugation (1300 x g for 5 min). The cells stained with anti-GLUT1 were resuspended in 200 μ L PBS and, on the other hand, the cells stained with anti-GLUT3 were incubated with anti-mouse secondary antibody conjugated to PE fluorochrome (1:100, Santa Cruz Biotechnology, sc-3738). After 20 minutes of incubation, the cells were washed with PBS by centrifugation (1300 x g for 5 min) and resuspended in 200 μ L of PBS. The cells were then excited using for this purpose a wavelength of 485/20 nm. For fluorescence detection, it was used an emission wavelength of 590/35 nm using a multi-detection plate reader.

Evaluation of PFK-1L, MCT4 and LDH expression by Western Blotting

Total proteins were extracted from HCC cells using RIPA buffer [150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, pH 8 and 1 mM EDTA]. The protein concentration was determined by the bicinchoninic acid assay (BCA™ Protein Assay kit, Pierce). 20 μ g of each sample were denatured at 100°C for 5 minutes and were fractionated on 12.5% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) gel at 160 V for 1 h. The separated proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, UK), previously activated with methanol and BioRad electrotransfer buffer, at 750 mA for 90 minutes. Next, the membrane was blocked for 1 h in a 5% non-fat milk solution at room temperature. Membranes were incubated overnight at 4°C with rabbit anti-PFK-1L (1:500, Santa Cruz Biotechnology, sc-292523), rabbit anti-MCT4 (1:1000, Santa Cruz Biotechnology, sc-50329), rabbit anti-LDH (1:10000, Abcam, Ab52488) primary antibodies. A mouse monoclonal antibody anti-tubulin (1:10000, Sigma, T9026) was used for protein loading control in all WB analysis. Goat anti-rabbit IgG-AP (1:40000, Santa Cruz Biotechnology) or goat anti-mouse IgG-AP (1:40000, Santa Cruz Biotechnology) were used as secondary antibodies. Membranes were incubated with Enhanced chemiluminescent (ECL) substrate (GE Healthcare), a fluorescent substrate for peroxidase-based detection, for up to 5 minutes and detected with the Molecular Imager FX Pro plus Multimager (Bio-Rad), according to standard methods. Band densities were obtained according to standard methods using the Image Lab 5.1 Software (Bio-Rad) and normalized by division with the respective alpha-tubulin band density. Results are presented as fold variation relative to control.

Statistical analysis

The statistical significance of differences between the treatment with hAMPE and control was assessed by unpaired t test and the differences between cell lines was assessed by two-way ANOVA (GraphPad Software). All experimental data are shown as mean $\pm$ SEM (n = 5 for each condition), significant differences were considered when $p < 0.05$.

Results

hAMPE induced increase glucose uptake in HCC cell lines

In order to determine whether hAMPE induced alterations in glucose consumption on Hep3B2.1-7, HepG2, and Huh7 cell lines, we resorted to the use of the ^1H NMR technique. Figure 2A shows the results obtained regarding the glucose consumption in Hep3B2.1-7, HepG2, and Huh7 cell lines, after 24 h of incubation in the presence of $1\text{ }\mu\text{g}/\mu\text{L}$ of hAMPE. All results were normalized to the respective control, i.e., cells from each cell line not treated with hAMPE (medium alone). It was verified a significant increase of glucose consumption in Hep3B2.1-7, HepG2, and Huh7 cell lines after treatment with hAMPE when compared with control. The increase of glucose was 17.6 ± 3.9 vs. 6.3 ± 1.9 , 15.7 ± 4.0 vs. 5.13 ± 1.5 , and 49.3 ± 7.7 vs. 14.3 ± 4.1 pmol/cell in Hep3B2.1-7, HepG2, and Huh7 cell lines, respectively. Besides, it was observed a significant increase of glucose consumption between Huh7 cell line after treatment with hAMPE when compared with others two types of cell lines.

hAMPE altered GLUT1, GLUT3 and PFK-1L expression

The role of hAMPE in the regulation of glycolytic metabolism was evaluated through the analysis of GLUT1, GLUT3 and PFK-1L expression levels in the HCC cell lines under study, Hep3B2.1-7, HepG2 and Huh7. The uptake of glucose in cells was performed through the expression of GLUTs. The protein levels of GLUT1 and GLUT3 are shown in Figure 2B and 2C, respectively. The results show that the treatment with hAMPE increased, respectively, 12.2% and 11.3% the expression of GLUT1 in Hep3B2.1-7 and Huh7 cells, relatively to control condition, which was considered equal to 1. On the other hand, the same treatment was not able to induce statistically significant changes in the expression of GLUT1 on HepG2 cell lines, but an increase of 13.3% in GLUT3 expression was detected when compared to control (Figure 2B and 2C). The treatment with hAMPE decreased 9% the expression of the GLUT3 in Huh7 cell line. However, the same experimental conditions were unable to alter the expression of GLUT3 in Hep3B2.1-7 cells, as can be seen in Figure 2C.

PFK-1L is one of the most important regulatory enzymes of glycolysis. Thus, we measured the expression of PFK-1L after the treatment with hAMPE. The Figure 2D and 2E represent the results of PFK-1L expression in three cell lines of the HCC. The expression levels of this enzyme were increased in all cell lines when compared to control. The increase was 1.7 ± 0.3 , 1.7 ± 0.3 and 3.9 ± 0.9 in Hep3B2.1-7, HepG2, and Huh7, respectively. As observed in glucose consumption, the Huh7 cell line presents a significant increase of PFK1L expression with hAMPE treatment when compared with others two types of cell lines.

hAMPE decreased lactate production in Huh7 cell line

In order to determine if hAMPE induced alterations of lactate production in Hep3B2.1-7, HepG2, and Huh7 cells, we resorted to the use of the ^1H NMR technique. Figure 3A represents the results obtained regarding the lactate production in Hep3B2.1-7, HepG2, and Huh7 cell lines, after 24 h of incubation in the presence of $1\text{ }\mu\text{g}/\mu\text{L}$ of hAMPE. All results were normalized to the respective control. The treatment with hAMPE was not able to induce statistically significant changes in lactate production in Hep3B2.1-7 (27.8 ± 3.9 vs. 18.8 ± 1.7) and HepG2 (4.5 ± 1.1 vs. 6.0 ± 2.2) cell lines when compared to control, although there was a tendency of increased in the Hep3B2.1-7 cells. However, a significant decrease (23.1 ± 1.8 vs. 35.2 ± 4.1 pmol/cell) in the levels of lactate in Huh7 cell line was detected. In addition, it was observed significant

differences between the cell lines: a significant increase of lactate production in Hep3B2.1-7 and Huh7 cells with hAMPE treatment when compared with HepG2 cells.

hAMPE induced increase LDH and MCT4 expression in Hep3B2.1-7 cell line

In order to determine whether hAMPE induce alterations in the expression of LDH and MCT4 on HCC cell lines, the expression of LDH and MCT4 were evaluated by Western blot. The results in Figure 3B and 3C shows the expression of LDH and MCT4, respectively, for Hep3B2.1-7, HepG2, and Huh7 cell lines, after 24 h of incubation in the presence of 1 $\mu\text{g}/\mu\text{L}$ of hAMPE. It was observed that the treatment with hAMPE significantly increased the expression of LDH in Hep3B2.1-7 and HepG2 cell lines (1.5- and 1.3-fold variation relative to control, respectively). No significant differences were observed in Huh7 cells, but there is a little positive trend (1.1-fold variation) in the expression of LDH. Regarding the expression of MCT4, which is lactate transporter required for lactate export in highly glycolytic cells, it was verified that hAMPE treatment significantly increased the expression of MCT4 in Hep3B2.1-7 (3.8-fold variation relative to control). On the other hand, there was no significant differences in the expression of MCT4 in HepG2 and Huh7 cell lines. A representative immunoblots for antibodies against LDH and MCT4 are shown in Figure 3D.

Discussion

HCC represents a major public health problem worldwide since it is a major cause of morbidity and mortality. HCC has a poor prognosis and it is associated with a high mortality rate, mainly due to the advanced stage at which most patients are diagnosed [2, 19]. Thus, it is urgent to find novel approaches of treatment, and in the last de decade, some research groups have pointed out the hAM as a potential therapy for cancer [11, 13, 20, 21]. Most of studies, which evaluated the application of hAM in the treatment of cancer, were performed using the amnion-derived cells, hAMCs or hAECs, or its conditioned medium. Considering the attractive properties of the amniotic membrane, in an innovative way, our research team has decided to evaluate the effect of hAMPE in cancer therapy. Our group demonstrated that the hAMPE can inhibit the metabolic activity of some human cancer cell lines, being the treatment highly selective [13]. Considering the human HCC cell lines evaluated, Hep3B2.1-7, HepG2, and Huh7 were highly responsive to hAMPE treatment, since its metabolic activity was inhibited [13]. Since it was demonstrated that hAMPE effectively inhibits the metabolic activity in Hep3B2.1-7, HepG2, and Huh7 cell lines, our research group has been investigating the mechanisms underlying the effect of hAMPE in HCC cells. Until now, it was demonstrated that hAMPE can induce selective cytotoxicity and cell death, and that the hAMPE treatment decreased the metabolic activity, protein and DNA content in a dose and time-dependent manner in HCC cell lines [22]. Although the hAMPE seems to be helpful in the therapy of HCC, the molecular mechanisms involved remain unknown. In the last years, it has been studied the relationship between the glycolytic metabolism and cancer. It is already known that tumor cells have metabolic changes that promote the evasion, growth and survival of cancer cells. One of the main

metabolic changes is described as the “Warburg effect”, which is based on the enhanced glycolytic metabolism of tumor cells even in the presence of low oxygen levels [7]. Considering that the HCC is usually under hypoxic conditions, the effect of Warburg is further favored, resulting in an elevated rate of glycolysis [23]. This dependence of glycolysis provides a biochemical basis for the design of new therapeutic strategies that preferentially kill cancer cells by the inhibition of glycolysis [24]. Taking into account that hAMPE inhibits the metabolic activity of HCC cells, the main aim of this work was to evaluate the effect of hAMPE treatment in glycolytic metabolism.

The glycolytic pathway starts with glucose uptake from the extracellular medium, and it is well known that HCC cells present a high glucose uptake [25]. Here, our results showed that the treatment with hAMPE increased the glucose uptake in all HCC cell lines, being even more pronounced in Huh7 cells than in Hep3B2.1-7 and HepG2 cells. This result can be explained by our previous research work, demonstrating that the effect of hAMPE in cell viability was less extensive in Huh7 when compared to Hep3B2.1-7 and HepG2 cells [14]. Altogether, these results suggest that the lower effect of hAMPE treatment in viability of Huh7 cells may be due to higher glucose uptake.

HCC cell lines uptake glucose from the extracellular medium through specific transporters, which are located in the plasma membrane. Several studies have reported changes in the expression and activity of GLUTs in cancer cells, where these changes are correlated with the high glucose demand that is essential to cancer cell growth and survival [26, 27]. GLUT1 and GLUT3 have altered expression in HCC, and are usually considered biomarkers of tumor differentiation and prognosis [26, 28, 29].

Therefore, and considering that glucose consumption increased in response to hAMPE treatment, we analyzed the GLUT1 and GLUT3 protein expression. Our results showed an increase of GLUT1 expression in Hep3B2.1-7 and Huh7 cell lines, but not in HepG2. Regarding HepG2 cells, it was detected increased GLUT3 expression in response to hAMPE treatment. Taken together, these results suggest that glucose uptake in Hep3B2.1-7 and Huh7 cells may be mediated by the GLUT1 protein, whereas in HepG2 can be by GLUT3.

An important controlling step of the hepatocellular glycolytic flux is the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate by PFK-1L enzyme. This is the first irreversible reaction in glycolysis, and it has been suggested that PFK-1L expression may be altered in tumor cells [30]. Our results suggest that glucose was actively metabolized in all cell lines because it was observed an increase of PFK-1L expression in all cell lines. The expression levels of PFK-1L were higher in Huh7 than in Hep3B2.1-7 and HepG2 cell lines. These results are in accordance with the glucose uptake in Huh7 cells, suggesting that it presents a higher glycolytic flux when compared with Hep3B2.1-7 and HepG2 cell lines. Considering the Warburg effect in cancer cells, it would be expected an increase in lactate production due to high glycolytic metabolism. Thus, we have decided to quantify the levels of lactate in HCC cell lines in response to hAMPE treatment. Our results showed a significant decrease of lactate levels in Huh7 cells, but there are no significant differences in lactate production on Hep3B2.1-7 and HepG2 cell lines. These results suggest that, even in the environment with high glucose uptake, the

hAMPE does not favor the lactate production, and may even lower it in Huh7 cells. This means that pyruvate, which is formed at the end of glycolysis, can be channeled to oxidative phosphorylation pathway in mitochondria.

Lactate production from substrates derived from glucose metabolism occurs via LDH, which catalyzes the conversion of pyruvate to lactate [8]. Some studies have shown that this enzyme is overexpressed, in tumor cells [31]. Thus, the effect of hAMPE in LDH expression was evaluated. There are no significant changes in LDH expression in Huh7 cells after hAMPE treatment, which is in accordance with the decreased levels of lactate. On the other hand, a significant increase of LDH expression was found in Hep3B2.1-7 and HepG2 cell lines. Apparently, this result is unexpected because no changes were observed in lactate production in response to hAMPE treatment. However, it should be taken into account that LDH acts on reversible way [8], supporting the idea that hAMPE may increase the levels of pyruvate, which is transported to mitochondrion and metabolized in acetyl-CoA, favoring the oxidative phosphorylation. It is well described that monocarboxylate transporters plays an important role in the hyperglycolytic profile of solid tumors, allowing the maintenance of the high glycolytic rates by performing lactate efflux, and pH regulation by the co-transport with protons [8]. The primarily lactate transporter expressed in cells with high glycolytic capacity is the MCT4 [32]. Increased levels of MCT4 protein have been associated with tumor progression and are associated with poor prognosis of HCC [33, 34]. Thus, it was evaluated the effect of hAMPE in MCT4 expression. Overall and as expected, no change in MCT4 expression was observed in HepG2 and Huh7 cells. On the other hand, a significant increase of MCT4 expression in Hep3B2.1-7 cells was observed. This result was unexpectedly and is unclear considering that no change in lactate levels was detected in Hep3B2.1-7 cells.

Overall, our results showed that hAMPE trigger effects that seems to favor the Warburg effects, such as increased glucose uptake and GLUT expression, but on the other hand, counteracts the Warburg effect because the lactate levels decrease, or no change is detected, in HCC cells in response to hAMPE treatment. Considering the increased expression of PFK-1L in all cell lines treated with hAMPE, it is clear that hAMPE stimulates the glycolytic pathway, increasing the levels of pyruvate. One way to counteract the Warburg effect is to favor the conversion of pyruvate into acetyl-CoA, through the action of pyruvate dehydrogenase complex (PDC), in the mitochondrion. In fact, several studies suggest that the Warburg effect is favored in HCC due to the inhibition of PDC [35]. The activity of PDC is inhibited by the activity of pyruvate dehydrogenase kinase (PDK), which is overexpressed in HCC [35]. Taking into account the global effects of hAMPE in HCC cells, it is liable to speculate that hAMPE may favor the oxidative phosphorylation in mitochondrion of HCC instead of aerobic glycolysis. It is well described that the inhibition of PDK in HCC cells, including Hep3B2.1-7, HepG2 and Huh7 cells, reduce the lactate production, inhibits cell proliferation and stimulates apoptosis [35, 36]. All these effects are triggered by hAMPE treatment of HCC cells, supporting the idea that hAMPE may reverse the glycolytic phenotype and induce cell injury in HCC cells. However, more studies are needed in order to clarify the mechanisms underlying the effects of hAMPE in mitochondrion. In addition, and in accordance to previously reported by our research team [13, 14, 22], it is clear that hAMPE induces different metabolic changes in HCC cell

lines, suggesting that identifying the specific tumor-profile, such as genetic and metabolic-profile, will be crucial to clarify the usefulness of hAMPE in the treatment of HCC.

In conclusion, our results showed that treatment of HCC cell lines with hAMPE increase the glycolysis without changes in lactate production, suggesting that oxidative phosphorylation may be favored instead of aerobic glycolysis. Moreover, it was shown that hAMPE was able to differentially modify the metabolism of HCC cells, suggesting that the effect of hAMPE may be dependent on the metabolic and genetic characteristics of each cell line. However, more studies are required to clarify the effect of hAMPE in HCC cells, as well as to identify a panel of biomarkers in cells, in order to predict the effects triggered by hAMPE treatment.

AUTHORS' CONTRIBUTIONS

All authors participated in the conception and design of study. APA, SMR, ACM and CJM interpret and analyzed of the data; APA, SMR, ACM and MGA conducted the experiments; ACM, MGA, PFO, MFB and CJM critically read the article; and APA and SMR wrote the manuscript. APA and SMR contributed equally to this study.

Declarations

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DECLARATION OF CONFLICTING INTERESTS

The authors declare that they have no conflicts of interest with the contents of this article.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Figures

Figure 1

Schematic illustration of the experimental outline of our study. hAM: human amniotic membrane, hAMPE: protein extract from human amniotic membrane.

Figure 2

Effect of hAMPE (1 µg/µL) on substrate consumption of Hep3B2.1-7, HepG2 and Huh7 cells after 24h incubation from control. (A) Glucose consumption, (B) GLUT1 expression, (C) GLUT3 expression, (D) PFK-L protein levels and (E) illustrative western blot experiment. All results are normalized relatively to control (no hAMPE treatment), which was considered equal to 1. Graphs represent mean ± standard error of the mean (SEM) (n = 5 for each condition). Significant differences are indicated by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

Figure 3

Effect of hAMPE (1 $\mu\text{g}/\mu\text{L}$) on metabolite production of Hep3B2.1-7, HepG2 and Huh7 cells after 24h incubation from control. (A) Lactate production, (B) LDH and (C) MCT4 protein levels, and (D) illustration western blot experiment. All results are normalized relatively to control (no hAMPE treatment), which was considered equal to 1. Graphs represent mean $\pm$ standard error of the mean (SEM) (n = 5 for each condition). Significant differences are indicated by * ($p < 0.05$) and ** ($p < 0.01$).