Research in Molecular Medicine

Influence of Bilirubin, Hemin, Zinc Protoporphyrin, Glutathione, Curcumin, and Their Combinations as a Supplement to Support the Viability and Functionality of Pancreas and Liver Cells

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ABSTRACT

Background: Previous experiments have shown different responses of pancreas and liver cells to the culture medium. It has been revealed that the most important step in preserving primary pancreas and liver cells is selecting the appropriate supplements to support the viability and functionality of these cells.

Materials and Methods: Cultivation supplements were prepared by adding bilirubin, hemin, zinc protoporphyrin, glutathione, curcumin, and their combination in the pancreas and liver cell culture at a concentration of 1, 3, and 5 µM. Then, the survival rate and function of the pancreas and liver cells were evaluated. The function of pancreas cells was evaluated based on producing insulin and the function of liver cells was based on liver enzymes, including transaminases.

Results: We found that bilirubin, hemin, zinc protoporphyrin, curcumin, glutathione, and their combination as supplements can dose-dependently maintain pancreas and liver cells viability and functionality proven by increasing insulin secretion levels and transaminase enzyme activity. The strength of effects is displayed in the following order: bilirubin > combination of all compounds > hemin > zinc protoporphyrin > curcumin > glutathione.

Conclusion: This study shows that these compounds are suitable supplements with special biochemical properties. They provide optimal supplements for the culture media of pancreas and liver cells. They may fulfill a function in the antioxidant protection of pancreas and liver cells.

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Introduction

Liver and pancreas cells acquire different cell culture media as a supplement to support their viability and functionality. Biochemical compounds are elicited by critical inductive regulatory factors. Interest in the development has been fueled by the intense need for biochemical compounds in the culture of pancreas and liver cells. Studies in diverse model media have evolutionarily revealed conserved inductive regulatory factors that elicit the viability and functionality of liver and pancreatic cells and guide how to increase the viability and functionality of these cells. It is important to overcome these problems. Therefore, the 1st step is to create precise conditions that provide high viability and functionality cells and the exact use of culture media.

It is well known that liver is composed of hepatocytes, biliary epithelial cells, and several non-epithelial cells [1]. Previous studies have demonstrated that pancreas is an organ that has endocrine and exocrine compartments [2, 3]. Previous studies established that liver and pancreas tissues share many common characteristics of the basic embryonic structure and the adult functional properties [4]. In this context, a study describes the potential use of liver and pancreas primary cell culture as a valuable tool for the characterization of liver and pancreas function [5]. It is now clear that both the viability and functionality of these cells during culture are influenced by culture chemical composition characteristics. Despite many studies that have been concerned with the pancreas and liver primary cell culture, many problems regarding successful cell functionality should be solved. Therefore, to find the best chemical composition for the pancreas and liver primary cell type, it is necessary to do more research on the supplementation of chemical composition. The importance of carrying out this work is to search for the best chemical composition. In this regard, it has been indicated that heme oxygenase is an enzyme catalyzing the degradation of heme [6].

Increasing evidence suggests that heme oxygenase-1 induction may mediate cellular protection against oxidant injury in the tissues [7]. Based on the previous study, one possible mechanism of the protective role of heme oxygenase-1 induction may be due to the antioxidant action of bilirubin [8]. It seems that hemin is a relatively strong inducer of heme biosynthesis [9-11]. It appears that un-conjugated bilirubin exerts a strong anti-oxidant activity [10]. Glutathione is a tri-peptide that plays a major role in erythrocytes and hepatocyte cells [11]. It is important to emphasize that hemin is used to treat acute porphyria and thalassemia intermedia [12]. It has been established that hemin is a relatively strong inducer of heme biosynthesis among various chemical inducers [13-15]. Variables such as suspension solution and cell density are important to the success of the cell delivery approach [16]. The delivery stage is pivotal for the therapeutic outcome as well as for laboratory isolation of the entrapped cells [17]. Researchers have reported the inhibitory role of proteins in liver and pancreatic cells [18].

Cell culture media generally comprise an appropriate source of supplements that regulate cell survival and growth. The presence of special compounds in the culture medium plays a crucial role in the activity and regulation of several biochemical pathways. It is important to note that the medium interacts with cells to regulate diverse functions, including survival and functionality. In this regard, the composition of the culture supplements which probably play a biochemical role may be important. Greater understanding in this area may help know signaling interactions between cells and the content of the culture medium. Biochemical compounds such as bilirubin, hemin, zinc protoporphyrin, curcumin, and glutathione have drawn considerable attention as a supplement to support the viability and functionality of pancreas and liver cells due to their low cost, excellent pharmacological activities, and lesser side effects. Despite its supplement properties, the experimental application of mentioned compounds as a supplement has been limited due to their fixed chemical stability, and low bioavailability. In an attempt to overcome these limitations, we prepared bilirubin, hemin, zinc protoporphyrin, curcumin, and glutathione as a supplement to check and assess the viability and functionality of pancreas and liver cells with their presence.

The purpose of the study and the importance of this study was to explore the effects of the presence or absence of bilirubin, hemin, zinc protoporphyrin, curcumin, glutathione, and their combination as media supplements on the viability and functionality of pancreas and liver primary cells. This study provides evidence of interactions between primary cells and their surrounding biochemical compounds in the culture medium.

Materials and Methods

Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum, penicillin-streptomycin, penicillin, streptomycin, and trypan blue were obtained from Gibco. Zinc protoporphyrin, bilirubin, curcumin, hemin, and glutathione were obtained from Sigma Aldrich Co, Germany. Glucose oxidase/peroxidase reagent was purchased from Sigma, Ger-

many. Phosphate-buffered saline (PBS) with pH 7.4 was obtained from Gibco. Mercodia Rat Insulin ELISA was purchased from Mercodia, Sweden. Ficoll-Paque™ premium was obtained from Pharmacia Biotech (Uppsala, Sweden). The Transaminase enzymes (ALT/GPT, AST/GOT) kit was purchased from Pars Azmoon Co. (Tehran, Iran).

Culture media preparation

To make a 5-µM bilirubin solution prepared as a sterile suspension, 29.2 mg of bilirubin was mixed with 100 mL of benzene solution. To make a 5-µM hemin solution, 32.55 mg of hemin was dissolved in 100 mL of 5 mM NaOH. To make a 5-µM curcumin solution, 18.4 mg of curcumin was dissolved in 100 mL sterile ethanol (95%). To make a 5-µM zinc protoporphyrin solution, 31.3 mg of zinc protoporphyrin was dissolved in 100 mL sterile ethanol (95%). To make a 5-µM glutathione solution, 15.35 mg of glutathione was dissolved in 100 mL distilled water. All solutions were incubated at 37°C in a water bath for 25 minutes. Also, the above-mentioned solutions were prepared as 1 and 3 µM concentrations through dilution of solutions.

Animals

Twelve male rats with an average weight of 190 to 230 g were included in the present experimental study. The animals were housed in a temperature-controlled room (23°C±2°C). The standard light cycle was 12 hours light followed by 12 hours dark. Animals were given tap water and standard rat chow. All procedures involving animals were carried out according to the standard guidelines.

Pancreas and liver primary cells were isolated according to previously described methods with some modifications [19]. Briefly, hepatocytes were obtained from rats using collagenase protocol. Purification of cell suspension was done utilizing centrifugation over Percoll. The rats were used as pancreas donors after anesthesia. The pancreas was isolated and cut into small tissue blocks. Tissue blocks were cultured at 37°C in a 5% CO_2-air humidified atmosphere. Submandibular glands were dissected. Cell suspensions were prepared by mincing and enzymatically dissociated by adding collagenase. The tissue cell suspension was filtered with a mesh and plated in plates. Pancreas and liver primary cells were cultivated in DMEM media (Gibco) supplemented with 10% fetal calf serum and 1% penicillin–streptomycin solution (10000 IU/mL penicillin; 10000 mg/mL streptomycin). Cell cultures were well kept in a humidified incubator at 37ºC, 5% CO_2. In passage 3, the cells were detached by a usual trypsin protocol, counted, and distributed in 96-well plates for trypan blue assay. Pancreas and liver cells were co-incubated with a solution of bilirubin, hemin, zinc protoporphyrin, curcumin, and glutathione and their combinations at different concentrations (1, 3, and 5 µM). After 48 hours, culture media was gently removed from each well to avoid cell layer scratch; the wells were washed with 500 µL of PBS and then cell count and trypan blue assay were performed to assess cell proliferation rate and viability of pancreas and liver cells in the presence or absence of bilirubin, hemin, zinc protoporphyrin, curcumin, glutathione, and their combinations.

Cell/well concentration

After 48 hours of culture, the cells were harvested. The effects of various cell concentrations were evaluated with bilirubin, hemin, zinc protoporphyrin, curcumin, glutathione, and their combinations at concentrations of 1, 3, and 5 µM. Each concentration was applied in triplicate.

Assessment of cell viability

All cells were cultured in a humidified 5% CO_2 incubator at 37ºC. Incubation was performed at 37°C for 48 hours in the dark. Initial cell viability and the sample mean viability were quantified using trypan blue. Cell viability was determined according to previously described methods. Cell viability was expressed as a percentage compared to the control. Cell count was carried out by a light microscope.

Assessment of cell functionality

Initial pancreas cell functionality was quantified using insulin secretion measurements. Insulin level was determined according to a previously described method [20, 21]. Also, initial liver cell functionality was quantified using transaminase enzyme level measurement. Transaminase enzyme activity was determined according to previously described methods [22]. Cell functionality was expressed as a percentage compared to the control.

Light microscopy

For light microscopy, the pancreas and liver cells were put on glass microscopy slides.

Statistical analysis

Statistical analysis was performed using SPSS software version 18. $P<0.05$ were considered significant. All values were expressed as the Mean±SEM. The sample size was based on the outcome of previous studies. Experiments were performed three times.

Results

In this study, the protection and improvement of pancreas and liver cell viability and its functionality were investigated in the presence or absence of bilirubin, hemin, zinc protoporphyrin, curcumin, glutathione, and their combinations for the 1st time.

The influence of bilirubin

After exposure to bilirubin for 48 hours, cell viability was significantly higher in bilirubin-treated pancreas and liver cells compared to untreated ones (95.63%±2.41%; 93.31%±1.32% vs 73.51%±2.04%; 75.16%±1.93%), respectively. Also, the cell count was significantly higher after exposure to 5 µM bilirubin for 48 h compared to untreated ones ($8.39\pm2.61\times10^5$/mL; $6.72\pm2.45\times10^5$ vs $3.45\pm1.35\times10^5$; $3.71\pm1.93\times10^5$/mL), respectively.

The influence of zinc protoporphyrin

After exposure to zinc protoporphyrin for 48 h, cell viability was significantly higher in zinc protoporphyrin-treated pancreas and liver cells compared to untreated ones (81.37%±3.48%; 89.62%±4.56% vs 68.58%±2.21 %;65.12%±1.85%), respectively. Also, the cells counts were significantly higher after exposure to 5 µM zinc protoporphyrin for 48 h compared to untreated ones ($6.07\pm2.41\times10^5$/mL; $4.98\pm1.98\times10^5$/mL vs $3.22\pm1.17\times10^5$; $3.31\pm1.49\times10^5$/mL), respectively.

The influence of hemin

The influence of hemin on the viability of pancreas and liver cells was determined after hemin treatment. The cell count was significantly higher after exposure to 5 µM hemin for 48 h compared to untreated ones ($6.24\pm3.12\times10^5$ /mL; $5.28\pm1.55\times10^5$/mL vs $3.37\pm1.38\times10^5$; $3.71\pm2.14\times10^5$/mL), respectively. Also, liver and pancreas cell viability was significantly higher after exposure to hemin compare to untreated ones (89.55%±2.31%; 90.03%±2.44% vs 71.34%±2.21%; 66.45%±2.13%), respectively.

The influence of curcumin

The influence of curcumin on the viability of pancreas and liver cell counts was determined after curcumin treatment. The cell count was significantly higher after exposure to 5 µM of curcumin for 48 hours compared to untreated ones ($5.93\pm0.35\times10^5$ /mL; $3.35\pm0.42\times10^5$ /mL vs $3.16\pm0.71\times10^5$; $3.19\pm0.27\times10^5$/mL), respectively. Also, liver and pancreas cell viability was significantly higher after exposure to curcumin compare to untreated ones (82.12%±2.27%; 89.63%±1.99% vs 69.18%±2.71%; 65.26%±1.86%), respectively.

The influence of glutathione

The influence of glutathione on the viability of pancreas and liver cells was determined after glutathione treatment. The cell count was significantly higher after exposure to 5 µM glutathione for 48 hours compared to untreated ($6.04\pm2.47\times10^5$/mL; $3.26\pm1.72\times10^5$/mL vs $3.01\pm1.15\times10^5$; $3.11\pm1.43\times10^5$/mL), respectively. Also, liver and pancreas cell viability was significantly higher after exposure to glutathione compare to untreated (80.12%±1.46%; 86.51%±1.97% vs 63.16%±2.11%; 63.26%±1.73%), respectively.

The influence of the combination of all compounds

The influence of the combination of all compounds on the viability of pancreas and liver cell counts were determined after the combination of all compounds treatment. The cell count was significantly higher after exposure to a 5-µM combination of all compounds for 48 hours compared to untreated ones ($7.38\pm1.37\times10^5$/mL; $5.49\pm1.63\times10^5$/mL vs. $3.15\pm1.35\times10^5$; $2.61\pm1.23\times10^5$ / mL), respectively. Also, liver and pancreas cells viability was significantly higher after exposure to the combination of all compounds (93.12%±1.31%; 92.45%±2.26% vs 72.42%±2.21%; 71.35%±1.78%), respectively.

Insulin levels were elevated after exposure to 1, 3, and 5 µM concentrations of bilirubin, hemin, zinc protoporphyrin, and their combinations, as well as curcumin and glutathione for 48 hours in a dose-dependent fashion in pancreas cells, compared to control cells (Figure 1).

Alanine aminotransferase activity was elevated after exposure to1, 3 and 5 µM bilirubin, hemin, zinc protoporphyrin, the combination of all these compounds, as well as curcumin and glutathione for 48 h hours in a dose-dependent fashion in the liver cell, compared to control cells (Figure 2).

Aspartate aminotransferase activity was elevated after exposure to 1, 3, and 5 µM concentrations of bilirubin, hemin, zinc protoporphyrin, a combination of all compounds, as well as curcumin and glutathione for 48 hours in a dose-dependent fashion in liver cells, compared to control cells (Figure 3).

The results following pancreas and liver cells incubation with various concentrations of bilirubin, hemin, zinc

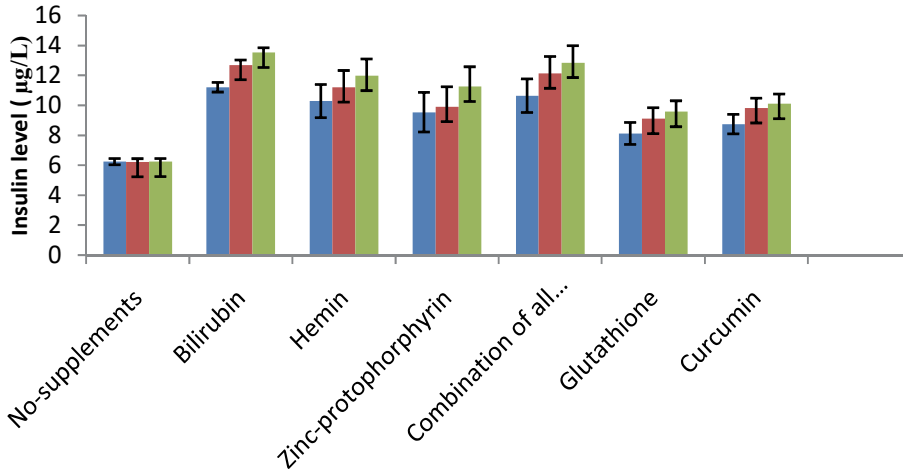

Figure 1. Insulin level in the presence or absence of supplements in pancreascells

Columns 1, 2 and 3 after exposure, (the concentration of each compound used in different treatments: 1, 3 and 5µM) of no–supplement, bilirubin, hemin, zinc-protophorphrin, combination of all compounds, glutathione and curcumin concentration for 48 hours,respectively. Experiments were performed three times.

Plane 1: No supplement; Plane 2: Bilirubin; Plane 3: Hemin; Plane 4: Zinc protoporphyrin; Plane 5: The combination of all compounds; Plane 6: Glutathione; Plane 7: Curcumin. Concentrations in each plane: Column 1: 1 µM; Column 2: 3 µM; Column 3: 5 µM. P<0.05.

protoporphyrin, glutathione, and a combination of all these compounds showed a clear influence on pancreas and liver cells functionality and viability. The strength of effects is displayed in the following order: bilirubin > combination of all these compounds > hemin > zinc protoporphyrin > curcumin > glutathione. These distinctions are likely illustrated by the different chemical types of compounds. In addition, different stimuli of pancreas and liver cells by these compounds could produce different responses at the cellular and molecular levels, and may therefore result in different cell reactions. The graphic design was demonstrated (Figure 4).

Discussion

In general, the choice of cell culture supplement significantly affects the success of cell culture experiments. In this regard, it is well known that finding good cell-cultured supplements is extremely important for the overall performance of primary cell cultures such as

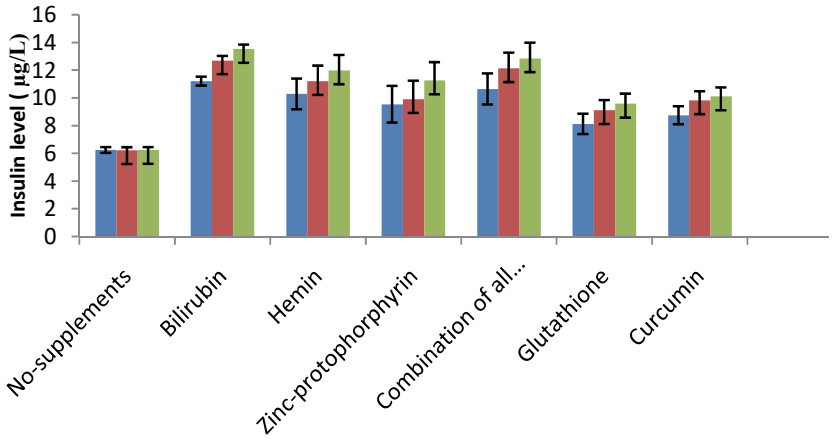

Figure 2. Alanine aminotransferase activity in the presence or absence of supplements in liver cells

Columns 1, 2 and 3 after exposure (the concentration of each compound used in different treatments: 1, 3 and 5µM) of no supplement, bilirubin, hemin, zinc-protophorphrin, combination of all compounds, glutathione and curcumin concentration for 48 hours, respectively. Experiments were performed three times.

Plane 1: No supplement; Plane 2: Bilirubin; Plane 3: Hemin; Plane 4: Zinc protoporphyrin; Plane 5: The combination of all compounds; Plane 6: Glutathione; Plane 7: Curcumin. Concentrations in each plane: Column 1: 1 µM; Column 2: 3 µM; Column 3: 5 µM. P<0.05.

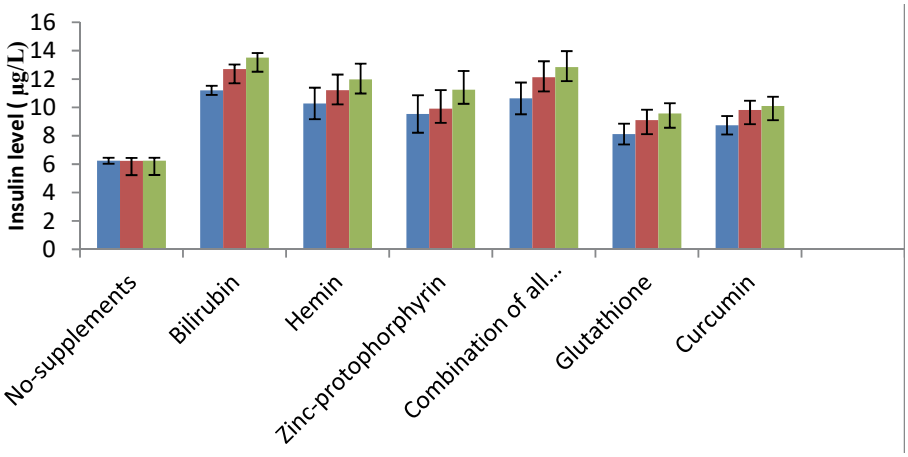

Figure 3. Aspartate amniotransferaseactivityin the presence or absence of supplements in liver cells

Columns 1, 2 and 3 after exposure (the concentration of each compound used in different treatments: 1, 3 and 5µM) of no-supplements, bilirubin, hemin, zinc-protophorphrin, combination of all compounds, glutathione and curcumin concentration for 48hours, respectively.Experiments were performed three times.

Plane 1: No supplement; Plane 2: Bilirubin; Plane 3: Hemin; Plane 4: Zinc protoporphyrin; Plane 5: The combination of all compounds; Plane 6: Glutathione; and Plane 7: Curcumin. Concentrations in each plane: Column 1: 1 µM; Column 2: 3 µM, Column 3: 5 µM. P<0.05.

pancreas and liver cells. Furthermore, many problems associated with the maintenance of the cell's viability and functionality should be solved to be successful in cell culture media. To solve the problems mentioned above, we tried to design a novel approach to maintain the ability and functionality of the pancreas and liver cells by adding bilirubin, hemin, zinc protoporphyrin, curcumin, glutathione, and a combination of all these compounds in the pancreas and liver cell culture media as supplements. The results of the current study demonstrated that these compounds were used as low-cost supplements with special biochemical properties providing an optimum primary pancreatic and liver cells supplements culture media compared to their respective untreated ones. Also, these findings are in line with the hypothesis that these chemical compounds may be metabolic regulators in these cells.

These results were sufficient to support the conclusion reached by us since insulin levels and the enzymatic activities of alanine aminotransferase and aspartate aminotransferase reach significant differences between treated and untreated groups. One possible mechanism of the positive simulative effect of these compounds on

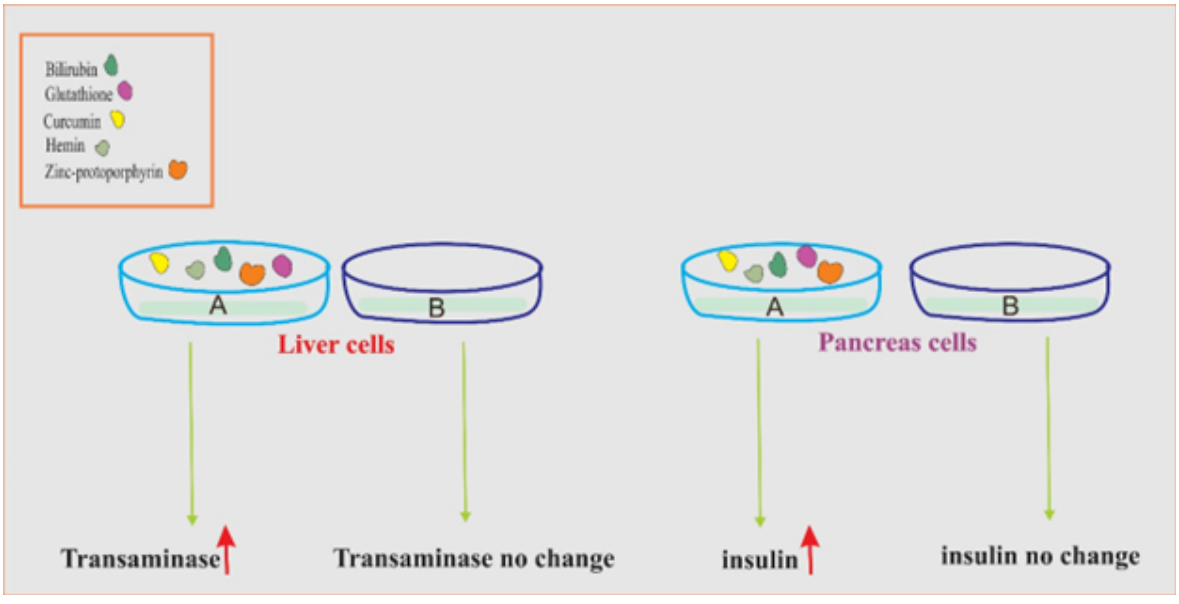

Figure 4. Graphic design

liver and pancreas cell viability may be due to the antioxidant activity of some of these supplements such as glutathione. Our results point out that each compound has a different effect on the improvement of cell viability and functionality. These different effects may be related to its solubility and antioxidant capacity. Whereas, the mechanism by which the improvement in cell viability and functionality with bilirubin is greater than others is not clearly understood. But, one possible explanation for this, bilirubin is proposed to have potential antioxidant activity, and protection, and also has biological properties which are essential for the culture media. It is also possible due to the rapid uptake of free bilirubin. Glutathione may protect liver and pancreas cells from oxidative stress and contributes to a favorable redox environment in cell culture media. It may also contribute to the metabolism of biochemical pathways, including the regulation of hormone secretion and biochemical metabolism.

Hemin may have a physiological role in the differentiation of some cells and the lipid peroxidation process. Hemin may enhance the differentiation of these cells. Curcumin may affect liver and pancreas cell survival. Curcumin has been shown to modulate signaling molecules. Curcumin could thus supply a useful constituent of dietary cell media aimed at protecting the cells. Our finding may explain these compounds' regulatory roles and their capacity to influence liver and pancreas cell signaling pathways. There are two main findings in our study that deserve attention.1st, these compounds are interesting with special biochemical properties and maintain the survival and function of pancreas and liver primary cells. These effects result in functional insulin increase from the pancreas and transaminase enzymes from the liver. 2nd, the observed effect of supplemented substances was dose-dependent. These results indicated that supplement value was important in the response of pancreas and liver primary cells.

In this context, researchers investigated the protective effects of some compounds, such as nicotinamide, lisofylline, and exendine-4 for improving the results of experimental and clinical on islet cells [23-25]. Gaps identified included scarcity of evidence for the effect on β-cell function and viability of extracellular vesicles from tissues such as muscle and liver [26]. Regenerative medicine technologies hold promises in a wide array of fields and applications, such as promoting the regeneration of native cell lines and augmenting the viability of existing ex vivo transplanted organs [27]. However, because of what was said about the mechanism of action of these compounds, many complications must be solved. To explore the role of these biochemical compounds, further studies using different cell concentrations and biochemical agents are necessary. Therefore, in our laboratory, new procedures will be conducted for exploration in a further study of the underlying mechanism.

Study limitations

Our study has some limitations. 1st, a major limitation of the study is that the concentration of other compounds was not measured in cell culture media. This fact does not allow us to derive conclusions about other compounds' changes concerning adding our supplemented substances. The 2nd limitation of our study was that the duration of the culture was short, thus, not allowing us to concentrate on the relationship between supplemented compounds and outcome for the longest time.

Conclusion

Collectively, the current results highlight the promising role of bilirubin, hemin, zinc protoporphyrin, curcumin, and glutathione as pancreas and liver cell culture media due to their interesting properties. However, further basic and clinical experimental investigations are needed to confirm these activities and their benefits. Consequently, the approach used in this study would be very important for understanding the role of the supplemented compound in these cell culture media and makes another slice of the puzzle into a picture of these supplements.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee for Research of Babol University of Medical Sciences (Code: 51,91/5/9, p/J/3/683).

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Authors contribution's

Experiments design and writing the manuscript: Durdi Qujeq; Performing the experiments: Roghayeh Pourbagher, Sadegh Fattahi and Zeinab Abediana. Data analysis: Sadegh Fattahi.

Conflict of interest

The authors declare no conflict of interest.

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