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Unexpected Relationship of Extracellular ATP with Intracellular Ca Concentration in HepG2 Cells

Armagan Caner[1,2]*, Nazmiye Bitgen[3] and Muge Gulcihan Onal[2]

[1] *Department of Biophysics, Erciyes University, Kayseri, Turkey*

[2] *Genome and Stem Cell Center, Erciyes University, Kayseri, Turkey*

[3] *Department of Chemical Technology, Technical Sciences Vocational School, Aksaray University, Aksaray, Turkey*

**Corresponding e-mail: armagan.caner@gmail.com*

ABSTRACT

The aim of our study is to examine the effect of extracellular ATP whether it is correlated with intracellular Ca concentrations ($[Ca^{2+}]i$) on human liver hepatocellular cells (HepG2). The extracellular ATP is responsible for regulating both cells signaling and cell functions. ATP maintains these effects through purinergic P2 receptors. The extracellular ATP promotes the release of Ca^{2+} from the Ca^{2+} stores to the cytoplasm in the cell and increases $[Ca^{2+}]$ I in the cell. In our study, various concentrations of ATP (10^{-3}M-10^{-7}M) were applied to HepG2 cells and incubated for 24 hours, 48 hours, and 72 hours. At these concentrations, the proliferation of cells and apoptosis of the cells was examined for 24 hours, 48 hours, and 72 hours. Similarly, cells with different ATP concentrations incubated for 24 hours and 48 hours were loaded with Indo 1FF AM calcium indicator to measure $[Ca^{2+}]i$. Surprising results were obtained, 10^{-6}M-10^{-7}M extracellular ATP was found to be more toxic than 10^{-3} M-10^{-4} M extracellular ATP, ($p<0.05$). Low concentrations of ATP also reduced $[Ca^{2+}]i$ for 24 hours and 48 hours of incubations ($p<0.01$). As a result, low concentration extracellular ATP is more toxic in HepG2 cells. At the same time, the extracellular ATP correlates with $[Ca^{2+}]i$.

Keywords: Extracellular ATP, Cancer cells, Imaging of intracellular Ca^{2+}

INTRODUCTION

It is well known that intracellular nucleotides, in particular, Adenosine triphosphates (ATP), function as a universal energy source [1]. Once ATP is released into the extracellular area, it acts as a primary signal involved in the control of various physiological and pathological mechanisms [2]. ATP is actively released from active or stretched cells, during inflammation, hypoxia or apoptosis, and passively from necrotic cells through ruptured cell membranes [3].

Many physiological activities are controlled by extracellular nucleotides. Specifically, ATP controls many activities, such as aging and development, controlling hormone secretion neurotransmission and neuromodulation [4-6]. In addition, extracellular nucleotides play an important role in thrombocyte activation and thrombus formation [7].

At the same time, ATP and other nucleotides are actively secreted during cancer growth and progression, they are actively released into the extracellular area or they play an important role as extracellular messengers [8,9].

In the extracellular area, ATP activates P2 purinergic receptors. P2 receptors consist of 2 subsets: P2X and P2Y receptors. P2X receptors (P2XRs) are plasma membrane channels activated only by ATP to mediate the flow of various cations such as Na^+, Ca^{2+}, K^+ in cells [10]. P2XRs can be sub-classed as fast-sensitive (P2X1R and P2X3R), slow-insensitive (P2X2R, P2X4R, and P2X7R) and non-functional under natural conditions (P2X5R and P2X6R). ATP binds to P2X1R, P2X2R, P2X3R, and P2X4R with low affinities. ATP has a high affinity to P2X7R [11].

The aim of our study was to determine the relation of extracellular ATP with intracellular Ca concentrations ($[Ca^{2+}]i$) in human liver hepatocellular cancer cells (HepG2). Different concentrations of ATP were applied to HepG2 cells and $[Ca^{2+}]i$ was visualized with Indo 1FF AM calcium indicator. Measured fluorescence intensities were evaluated as $[Ca^{2+}]i$.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The HepG2 cell lines were purchased from the American Type Culture Collection. The cells were cultured in an incubator containing 5% CO_2 at 37°C. The culture medium of DMEM F12 (21331020 Gibco) was supplemented with 10% FCS and 5% L-Glutamin-Penicillin (Penicillin (1-105 U/L) and L-Glutamine (2 mmol/l)). Periodically, the cells were harvested with trypsin (0.05%), EDTA (0.02%), and resuspended in medium. Trypan Blue staining was used to assess cell viability. The cells were counted microscopically by Thoma Slide.

Drugs and Reagents

Adenosine 5'-triphosphate disodium salt hydrate was obtained from Sigma (Cat. No. A7699). It was solved in sterile water and prepared a 10^{-1} M stock solution. It was stored at -20°C.

Cell Proliferation Assay

After trypsinization, the cells were plated in 1 mL of medium in 24-well plates at a density of 1.105-1.106 cells/well, depending on the length of the incubation period (24 hours, 48 hours, 72 hours). They were incubated in an incubator with 5% CO_2 at 37°C for overnight. After incubation, the cell culture medium was replaced with 1 mL/well medium containing increasing concentrations of ATP; 10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7} M. Each treatment was performed in duplicate. After 24 hours, 48 hours, and 72 hours of treatment, the cells were harvested for DNA measurement. Measurement of total DNA content which represent a number of cells was performed using the bisbenzimide fluorescent dye (Hoechst 33258 cat. no 14530 Sigma), as previously described [12].

Assessment of Apoptosis with Giemsa staining of HepG2 cells

HepG2 cells were plated in 1 mL of medium in 12-well plates. The cells were grown to 80% to 85% confluency in 12 well plates and then they were treated with ATP (10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7} M). Then, the cells were incubated at 37°C and with 5% CO_2 for 24 hours, 48 hours, and 72 hours. HepG2 cells were washed with PBS. They were fixed with cold methanol for 5 min. After methanol was discarded, the cells were air-dried. Fixed cells were treated with 6% Giemsa in Sorensen's phosphate buffer and incubated with this buffer for 10 min. The cells were washed 3 times with water. Cells were air-dried. They were visualized under an inverted light microscope by using a 40X objective. For each concentration of ATP, 1000 cells were counted by us. The number of apoptotic cells was determined. Apoptotic cells were recognized by their condensed and darker nuclear staining and changed morphology.

Measurement of Fluorescence Intensity for Intracellular Ca^{2+}

HepG2 cells were plated in 1 mL of medium in 6-well plates. The cells were grown to 90% to 95% confluency in 6-well plates and then they were treated with ATP (10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7} M). Then, the cells were incubated at 37°C and with 5% CO_2 for 24 hours and 48 hours. HepG2 cells were washed with PBS. After trypsinization, the cells were collected to the tubes according to applied ATP concentration. For imaging of calcium, calcium indicator Indo 1FF AM (cat. no. 17088 Sigma) was used. A buffer was prepared which contains Indo 1FF AM, KCl (cat. no. 12636 Sigma) and Hepes (cat. no. H4034 Sigma) (20 uM Indo 1FF AM, 160 mM KCl and 20 mM Hepes). HepG2 cells with different concentration of ATP were treated with this Indo 1FF AM buffer for 1 hour in a dark room with gentle stirring. The fluorescence microscopy was used to visualize the cells. Before microscopic studies, the cells were washed twice with KCI-Hepes buffer.

The cell solution was filled into microslides in the dark room. It is stimulated with a single wavelength and displayed at 2 emission wavelengths. So, it is stimulated at a wavelength of about 350 nm and the emission wavelength changes whether Indo 1FF AM with Ca^{2+} ion and Indo 1FF AM without Ca^{2+} ion. The emission wavelength is about 405 nm while Indo 1FF AM is connected to the Ca^{2+} ion and the emission wavelength is about 485 nm while the Ca^{2+} ion is free. Therefore, the samples were stimulated at a wavelength of 350 nm and photographs were taken at 405 nm and 485 nm wavelengths simultaneously. The fluorescence intensities were measured from images taken at both the 405 nm wavelength and the 485 nm wavelength of the cells by Fiji ImageJ software. For each sample, the fluorescence intensities of 25 cells were calculated at both wavelengths. These fluorescence intensities were proportioned ($R=F\lambda_{485}$ nm/$F\lambda_{405}$ nm). $[Ca^{2+}]i$ was evaluated according to the ratio of fluorescence intensities.

Statistical Analyses

All experiments were carried out at least 3 times but 2 best coherent results were considered. GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) was used for statistical analysis. Half-maximal effect concentrations (EC50) were calculated using log (agonist) vs. response (3 parameters) program in GraphPad Prism 5.0. For the comparative statistical evaluations, ANOVA test was used. When significant differences were found, a comparison between groups was made using the Newman Keuls test.

RESULTS

Proliferative Effects of Extracellular ATP

In our study investigating the effect of ATP on HepG2 cells, unexpected results were obtained. When we look at the differences in EC50 values of extracellular ATP at different incubation times, it was found that the concentration increased approximately 10 times every 24 hours. After 24 hours, 48 hours and 72 hours of incubation, the extracellular ATP significantly increased the growth of HepG2 cells in a dose-dependent manner with a mean EC50 of 4,3 10^{-5} M, 9,3 10^{-4}M and 4,3 10^{-3}M, respectively.

After 24 hours, 10^{-7} M and 10^{-6} M concentration of ATP significantly inhibited cell proliferation (Figure 1A) ($p<0.05$). But 10^{-5} M, 10^{-4} M and 10^{-3} M concentration of ATP had no statistically significant effect on HepG2 cells. There was an interesting point in these results that the high concentration of ATP had an effect similar to control.

After 48 hours, 10^{-4} M and 10^{-3} M concentrations of ATP significantly increased cell proliferation (Figure 1B.) ($p<0.05$). The 10^{-7} M, 10^{-6} M and 10^{-5} M concentration of ATP significantly inhibited cell proliferation ($p<0.05$). When the high concentrations were compared to the control group, there was not any difference between them. After 72 hours, there was not any significant difference between all concentration of ATP (Figure 1C).

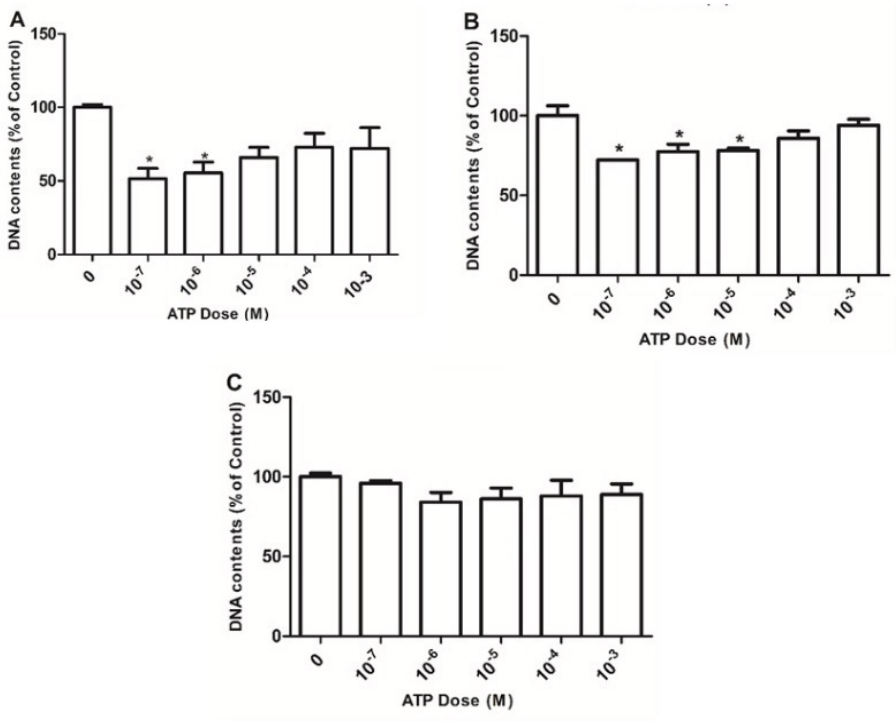

Figure 1 Effects of extracellular ATP (A-C) treatment on cell proliferation, as measured by total DNA content, using Hoechst 33258. HepG2 cell lines were incubated for (A): 24 hours; (B): 48 hours; (C): 72 hours without (control) or with the ATP indicated at different concentrations. Values are expressed as the percentage of control (untreated cells) and represent the mean ± SEM of at least 3 independent experiments in duplicate. ***p<0.001; **p<0.01; *p<0.05 versus control

Effects of Extracellular ATP on Apoptosis

Interestingly, in our study, the apoptotic effect of low concentrations of ATP was found to be higher than the high concentrations of ATP. After 24 hours of incubation, 10^{-7} M, 10^{-6} M, 10^{-5} M and 10^{-4} M concentration of ATP had a significant effect on apoptosis (Figure 2A). According to the control, 10^{-3} M ATP had no statistically significant difference in apoptosis. After 48 hours, there was a significant difference between control and all concentrations of ATP (Figure 2B). But, 10^{-7} M and 10^{-6} M concentration of ATP were more effective on apoptosis. When HepG2 cells with the valuable concentrations of ATP were incubated for 72 hours, all concentrations of ATP had a significant effect on apoptosis (Figure 2C). The 10^{-7} M concentration of ATP was more effective on apoptosis. Cells with necrosis were not observed under the microscope. In our study, extracellular ATP led to apoptosis in all concentrations of cells, and not to necrosis.

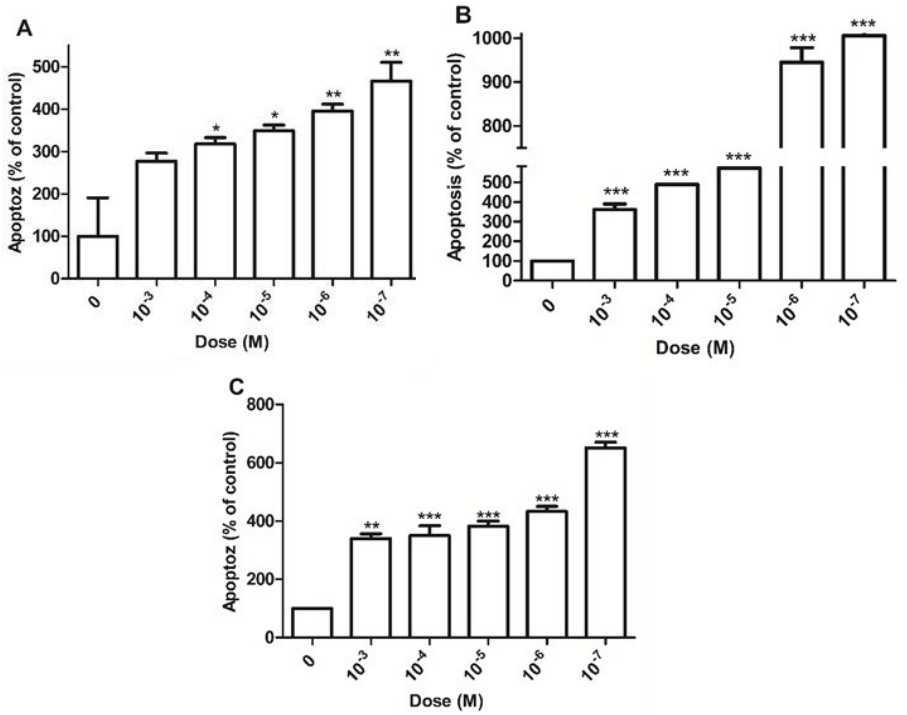

Figure 2 Effects of extracellular ATP treatment on apoptosis after (A): 24 hours; (B): 48 hours; (C): 72 hours in HepG2 cell lines. Induction apoptosis in HepG2 cancer cells was assessed by Giemsa staining. ***p<0.001; **p<0.01; *p<0.05 versus control

Effects of Extracellular ATP on Intracellular Ca^{2+}

After 24 hours and 48 hours of HepG2 cells treated with ATP at different concentrations, $[Ca^{2+}]i$ was marked with Indo 1FF AM which is known as calcium ion indicator. Their images under the fluorescence microscopy were taken as shown in Figure 3.

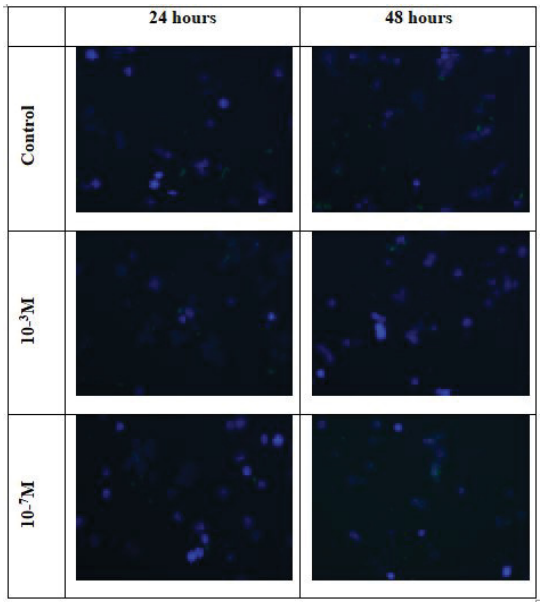

Figure 3 The pictures of intracellular Ca^{2+} for 24 hours and 48 hours

Since Indo 1FF AM is a radiometric calcium indicator, this indicator is displayed at different wavelengths when connected to the Ca^{2+} and at different wavelengths when not connected to the Ca^{2+}. In our study, the ratio of the fluorescence intensities of these 2 different wavelengths (R=F λ_{485} nm/F λ_{405} nm) was evaluated. As a result, 24 hours later, at 10^{-7} M and 10^{-6} M concentrations of ATP, $[Ca^{2+}]$i was also decreased significantly. After 48 hours, 10^{-4} M concentration of ATP decreased the $[Ca^{2+}]$i when compared to 24 hours. Depending on the dose and time, extracellular ATP affects $[Ca^{2+}]$I (Figure 4).

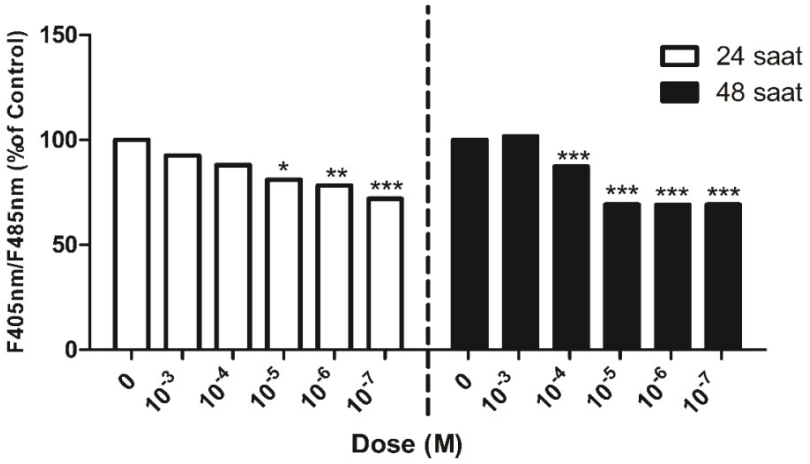

Figure 4 The ratio values of fluorescence intensity of Indo 1FF AM in the HepG2 cells with a valuable concentration of ATP after 24 and 48 hours. To measure intracellular calcium concentration, HepG2 cells were loaded Indo 1FF AM. ***p< 0.001; **p<0.01; *p<0.05 versus control

There was a correlation among the $[Ca^{2+}]$i of HepG2 cells treated with extracellular ATP, apoptosis and cell proliferation. After 24 hours incubation, there was a negative correlation with $[Ca^{2+}]$i ($r=-0.9636$, $p<0.01$). That is, after 24 hours, while the $[Ca^{2+}]$i of the HepG2 cells decreased, apoptosis occurred. There was a positive correlation between the $[Ca^{2+}]$i and cell proliferation after 24 hours incubation ($r=0.9448$, $p<0.01$). That is, $[Ca^{2+}]$i and the cell proliferation increased in direct proportion to each other. There was a negative correlation between the $[Ca^{2+}]$i and apoptosis after 48 hours incubation ($r=0.8627$, $p<0.05$). There was a positive correlation between $[Ca^{2+}]$i and cell proliferation after 48 hours incubation ($r=0.9568$, $p<0.01$).

DISCUSSION

In our study, when the data was analyzed, surprising results were obtained. When the data were evaluated according to the concentrations, extracellular ATP was found to be more toxic in low concentrations. At the same time, the $[Ca^{2+}]$ i were found to be decreased in the low concentrations of ATP when compared to control. In a breast cancer study, Jiang, et al., reported that the extracellular ATP has an inhibitory effect and that high concentrations of ATP increase breast cancer cell growth and migration [13]. Our study also showed that the extracellular ATP had an inhibitory effect on HepG2 cells, but this effect decreased in high concentrations. In contrast, in a study of human colon cancer cells, the extracellular ATP concentrations (≈10 mM) have been reported to suppress the development of Caco2 cells [14].

In the study by Amaral, et al., 18 hours and 24 hours 10 μM-100 μM ATP applied primary mouse hepatocellular cells have been shown to die 40% in their study [15]. Our results show that HepG2 cells treated with different concentrations of ATP, after 24 hours, the apoptosis rate was 35% to 40% at ATP concentrations between 0.1 μM and 100 μM. Likewise, after 48 hours, apoptosis of the same cells was 80% at ATP between 0.1 μM and 1000 μM. However, after 72 hours, this situation changes and the apoptosis rate was less than 48 hours. The cytotoxic effects can be performed by means of purinergic receptors known to be expressed on hepatocytes [16,17]. Xieve, et al., reported that extracellular ATP did not affect viability in primary mouse hepatocellular cells [18]. According to our results, cell proliferation was decreased by 40% in human liver hepatocellular cells treated with 0.1 μM and 1 μM ATP after 24 hours. After 48 hours, this ratio increases up to 60% at ATP concentrations between 0.1 μM and 100 μM. Xieve, et al., report that extracellular ATP does not directly cause cytotoxicity [6], whereas another study reports that extracellular ATP has cytotoxic effects in hepatic cells [18]. So, there is not any clear explanation for cytotoxicity. In this study, it can be interpreted that the low concentration of ATP is cytotoxic for HepG2 cells. Several studies have reported that ATP exhibits anti-cancer properties by binding to P2 purinergic receptors [19].

All P2X receptors are ATP receptors, and there is a significant difference with P2Y receptors capable of binding a number of nucleotides. The EC50 values of ATP are 1 μM to 10 μM for all P2X receptors, except for P2X7, which has the EC50 value of approximately 100 μM [20]. Our results are different than the study of Mammen, et al., [20]. According to our results, the EC50 value is 10 times higher for extracellular ATP in 24 hours incubation. The current carried by P2X1 or P2X3 receptors during the continuous exposure to ATP decreases by milliseconds, while desensitization may be much slower for P2X2, P2X4 and P2X7 receptors [21]. In addition, when ATP implementation is short, P2X2, P2X4 and especially P2X7 receptors have low permeability to large organic cations, but permeability increases when ATP implementation is prolonged [22].

ATP released from a necrotic cell was reported to bind to P2 receptors in a neighboring cell and increase the $[Ca^{2+}]$ i to sufficiently high levels to accelerate cell damage or even death [15]. However, in our study, the apoptosis rate is too high, not necrosis.

Another study reported that in the first 90 minutes, intracellular ATP levels were maintained, after the appearance of the first apoptotic cells, intracellular ATP levels eventually decreased [23]. The extracellular ATP concentration increases significantly during necrosis [24]. In our study, extracellular ATP was found to be effective in apoptosis of HepG2 cells. The extracellular ATP concentration increases during inflammatory responses, but it reaches maximum concentration in the 100 μM [15]. In addition, extracellular ATP has an inflammatory effect on immune cells. Amaral et al. Reported that the ATP signal during necrosis was effective in 2 ways; i) Increases the inflammatory response through the release of IL-1β and ii) exhibit hypersensitivity to ATP and directly causes hepatotoxicity due to the presence of increased $[Ca^{2+}]$i [15].

Our study is contradictory with literature that extracellular ATP decreases $[Ca^{2+}]$i. According to our results, $[Ca^{2+}]$i in the cells treated with 10^{-6} M and 10^{-7} M extracellular ATP were significantly decreased after 24 hours and 48 hours. After 48 hours, implementation of 10^{-4} M extracellular ATP decreased $[Ca^{2+}]$i.

However, in a study with human prostate cancer, 100 μM ATP was shown to increase $[Ca^{2+}]$i [25]. Similarly, in a study conducted with breast cancer, ATP implementation between 10^{-7} M and 10^{-3} M increased the $[Ca^{2+}]$i [26]. By extracellular ATP, activation of P2X receptors results in membrane depolarization. Then, in many cases, Ca^{2+} influx [27].

In our study, low extracellular ATP concentration decreased $[Ca^{2+}]$i. Grol, et al., reported the $[Ca^{2+}]$i after 240 seconds. In our study, the concentrations of 24 hours and 48 hours were evaluated. On the other hand, they observed higher and longer-lasting $[Ca^{2+}]$i elevations over 20 minutes with a higher ATP concentration. Grol, et al., showed that different ATP concentrations were transformed into different cellular signals [27].

CONCLUSION

In conclusion, our study showed that extracellular ATP had an inhibitory effect on human liver hepatocellular cancer cells. This inhibition effect of extracellular ATP was at low concentrations. High concentrations of ATP had an inducing effect on cells. At the same time, $[Ca^{2+}]i$ was reduced when low ATP concentrations were applied. More detailed molecular studies will be conducted to support the results of our study. Our study has suggested that the therapeutic effect of ATP in cancer cells will only occur when implemented at the right concentrations.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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