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RESEARCH ARTICLE

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Involvement of GSK3/β-catenin in the action of extracellular ATP on differentiation of primary cultures from rat calvaria into osteoblasts

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Abstract

Modulation of purinergic receptors play an important role in the regulation of osteoblasts differentiation and bone formation. In this study, we investigated the involvement of the GSK3/βcatenin signaling in the action of ATPγ-S on osteogenic differentiation of primary cell cultures from rat calvaria. Our results indicate that the cell treatment with 10 or 100 μM ATPγ-S for 96 h increase the cytoplasmic levels of β-catenin and its translocation to nucleus respect to control. A similar effect was observed after cell treatment with the GSK3 inhibitor LiCl (10 mM). Cell treatments with 4-10 mM LiCl significantly stimulated ALP activity respect to control at 4 and 7 days, suggesting that inhibition of GSK-3 mediates osteoblastic differentiation of rat calvarial cells. Effects comparison between ATP and LiCl shown that ALP activity was significantly increased by 10 μM ATPγ-S and decreased by 10 mM LiCl at 10 day of treatment, respect to control, suggesting that the effect of ATPγ-S was less potent but more persistent than of LiCl in stimulating this osteogenic marker in calvarial cells. Cell culture mineralization was significantly increased by treatment with 10 μM ATPγ-S and decreased by 10 mM LiCl, respect to control. In together, these results suggest that GSK3 inhibition is involved in ATPγ-S action on rat calvarial cell differentiation into osteoblasts at early steadies. In addition such inhibition by LiCl appear promote osteoblasts differentiation at beginning but has a deleterious effect on its function at later steadies as the extracellular matrix mineralization.

KEYWORDS
βcatenin, GSK3, osteoblast differentiation, purinergic receptors

Abbreviations: ADP, adenosine-5′-diphosphate; ADPβ-S, adenosine-5′-O-(2-thiodiphosphate); AKT, serine/threonine kinase Akt (also known as protein kinase B or PKB); ALP, alkaline phosphatase; ATP, adenosine-5′-triphosphate; ATPγ-S, adenosine-5′-O-(thiotriphosphate); BMP-3, -4 and -5, bone mophogenetic protein 3, 4 and 5; BSP, bone sialo protein; DAG, diacylglycerol; EDTA, ethylenediaminetetraacetic acid; ERK1/2, extracellular signal regulated kinases one and 2; GSK-3, glycogen synthase kinase-3; IP3, inositol trisphosphate; PBS, phosphate buffered saline; PI3 K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PI-PLC, phosphatidylinositol-specific phospholipase C; PKC, protein kinase C; PP2A, protein phosphatase 2A; TGF-β, transforming growth factor β; UTP, uridine-5′-triphosphate; NPP1, nucleotide pyrophosphatase/phosphodiesterase-1; PPi, inorganic pyrophosphate.

1 | INTRODUCTION

Adenosine-5′-triphosphate (ATP) can be released from cells via several mechanisms under both physiological and pathological conditions.[1–6] ATP, related nucleotides and adenosine have shown elicit a role as important local regulators of osteogenic differentiation and in the activation of bone remodeling.[7–9] They act as chemical messengers through activation of cell surface receptors, namely purinergic or P receptors.[10] P receptors are divided in two families, P1 and P2. The main agonist of P1 receptors is the nucleoside adenosine, whereas the P2 receptors family responds to nucleotides. Moreover, P2 family is subdivided into P2X (P2 × 1−7), ionotropic receptors (ligand-gated ion channels) which only respond to ATP, and P2Y (P2Y1, 2, 4, 6, 11-14), metabotropic/G protein- coupled receptors that are activated by adenine and uridine nucleotides.[11,12]

Recently, it has been reported up- or down-regulation of several P2 receptor subtypes, at gene and protein level, during human mesenchymal stem cells adipogenic and osteogenic differentiation. In addition, differentiation to one or other linage is directly influenced by both the application of P2 receptors agonists/antagonists and apyrase-induced nucleotide cleavage.[7,13,14]

Previously, we demonstrated that activation of PI3K/AKT signaling pathway by extracellular ATP stimulates cell proliferation and alkaline phosphatase (ALP) activity in neonatal rat calvarial primary cultures, suggesting purinergic signaling as positive modulator of the osteoblast maturation.[15] In addition, it was shown that increases in intracellular calcium concentration ($[Ca^{2+}]i$), stimulation of PI3K/Akt signaling pathway and members of the MAPKs such as ERK1/2, p38, and JNK1 through activation of P2Y2 receptors are involved in the stimulation of osteoblast proliferation by ATP and UTP.[15–17]

Extracellular nucleotides have been shown to induce differentiation of aortic valve cells into osteoblast phenotype.[18] ATP and UTP activation of Runx2, a key transcription factor for osteoblasts differentiation, has been observed in the osteoblast-like HOBIT cell line.[19] It has been suggested that the P2Y2 receptors inhibit the mineralization of osteoblasts in vitro.[20] However, our recent studies suggest that stimulation of P2Y2 receptors by ATP, UTP, or ATPγ-S enhances osteoblast differentiation and mineralization of rat calvarial primary cultures, which was associated with PI3K/Akt activation and gene expression increased of alkaline phosphatase (ALP), bone sialo protein (BSP), and bone morphogenetic proteins-2, -4, and -5 (BMP2-4-5).[21] In addition we demonstrated that an osteogenic medium containing 10 μM ATPγ-S and high calcium concentration (5.35 mM) enhance osteogenesis and mineralization by rat calvarial primary cultures, suggesting that purinergic signaling can favor the action of other osteogenesis inducers such as elevated extracellular calcium concentration.[22]

Glycogen synthase kinase-3 (GSK-3), a key regulator of glycogen metabolism, is a serine/threonine kinase, ubiquitously expressed as two closely related isoforms (GSK-3α and GSK-3β) in mammalian tissues, that regulates cellular functions including cell proliferation and differentiation.[23–26] GSK-3 is present in a protein complex integrated by axin, adenomatosis polyposis coli (APC), CK1, and β-catenin. GSK3- phosphorylates β-catenin, inducing its rapid degradation through the E3 ubiquitin/proteasome pathway.[27] Inhibition of GSK-3 prevents the phosphorylation of β-catenin. This leads to the stabilization, accumulation, and translocation of β-catenin from cytoplasm to nucleus and binding to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors to activate transcription of specific genes.[28–31]

GSK-3β deficiency affects bone development and regeneration in mice, suggesting that bone formation and regeneration in vivo are accelerated by inhibition of GSK-3β.[32] In fact, GSK-3 inhibitors stimulate osteoblastogenesis from mesenchymal stem cells in vitro.[33–36] Also it was suggested that local application of lithium (or other GSK-3 inhibitor) facilitates recovery from bone injury by promoting osteoblastogenesis and inhibiting osteoclastogenesis.[35]

Several kinases including PKA, PKC, p70S6 kinase, Akt, and others catalyze the phosphorylation and inactivation of GSK3.[31,37–41] It has been shown that activation of the PI3K/Akt pathway by G-protein coupled receptors inactivates GSK3, independently of the Wnt pathway, favoring β-catenin stability and translocation to nucleus.[42,43] As mentioned above, the PI3K/Akt signaling pathway has been implicated in ATP-induced stimulation of neonatal rat calvarial cells proliferation and their differentiation into mature osteoblasts.[15,21,22] GSK-3 phosphorylation and β-catenin nuclear translocation after P2Y receptor stimulation was reported in granule neurons.[44] However, involvement of GSK-3/β-catenin pathway in the modulation of osteoblasts proliferation and differentiation by extracellular ATP has not been studied yet.

We hypothesized that GSK-3 and β-catenin pathway may be involved in the ATP effects on osteoblasts differentiation.

As ATP is rapidly hydrolyzed to ADP, AMP, and adenosine by the action of extracellular enzymes,[10–12] its effect can be mediate by joint activation of multiple purinergic receptor subtypes of the P1 and P2 families.[10] The use of a nonhydrolyzable analogue of ATP, as ATPγ-S, helps reduce the spectrum of receptors involved since only some P2 receptor subtypes would be activated by this compound.[11,12] ATPγ-S is not the preferential agonist for P2X receptors family and between the P2Y groups, only a few receptor subtypes as P2Y2, P2Y4, and P2Y11, prefer nucleotides triphosphate as agonist.[12,45,46]

The aim of this study is to compare the effect of LiCl, an inhibitor of GSK-3,[29,47,48] with the action of ATPγ-S

treatment on growth and differentiation into osteoblasts of rat calvarial cells.

In this work, we obtained evidence that indicates, for the first time, that ATPγ-S stimulates cytoplasmic accumulation and nuclear translocation of β-catenin in neonatal rat calvarial cells. This effect is accompanied by increased cell osteogenic differentiation that involves PI3K/AKT activation, suggesting that GSK-3 inhibition mediated by AKT could be participating

2 | MATERIALS AND METHODS

2.1 | Materials

ATPγ-S, UTP, Alizarin Red, Ascorbic Acid, β-glycerophosphate and α-MEM (1.36 mM Ca^{2+}; $CaCl_2 . 2H_2O$ 0.2 g/L) were from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was from Natocor (Córdoba, Argentina). Alkaline phosphatase (ALP) activity kit was donated by Wiener Lab. (Rosario, Argentina). Antibody anti-βcatenin was donated by Paula Monje, PhD (The Miami Project to Cure Paralysis and Department of Neurological Surgery, University of Miami Miller School of Medicine, Miami, Florida). Neutral Red dye was donated by Paula Messina, PhD (Chemistry Department of Universidad Nacional del Sur, Bahía Blanca, Argentina). All other reagents used were of analytical grade.

2.2 | Cell isolation

Calvarial cells were obtained from 3 to 5-day-old neonatal rats. Animals were sacrificed by fast decapitation. All procedures were carried out in conformity with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) under protocol n° 012/2014 of Institutional Animal Care and Use Committee (CICUAE) of Universidad Nacional del Sur, Argentina. Briefly, calvarias were incubated in phosphate buffer saline (PBS) containing 4 mM EDTA at 37°C for two 10-min periods and the supernatants were discarded. Subsequently, calvarias were rinsed in PBS and submitted to digestion in PBS containing 200 U/mL collagenase for four 15-min periods. Cells released during the first digestion were discarded and those released during the subsequent digestions were spun down, collected and combined after centrifugation during 10 min at 1500 rpm. Then, cells were cultured at 37°C in α-MEM supplemented with 15% FBS, 1% penicillin, and streptomycin under humidified air (5.5% CO_2). After 24 h, the medium was replaced by α-MEM supplemented with 10% FBS, 1% penicillin and streptomycin and the cells were cultured until ~80% of confluence (2-3 days). Then the cells were frozen in liquid nitrogen until their use.

2.3 | Cell culture and treatment

Cells were thawed and seeded into 10 cm diameter glass Petri dishes and allowed to grow to 80% confluence. Then, cells were passed at a density of 10^4 cells/cm^2 and cultured for 3-4 days in α-MEM supplemented with 10% FBS, in a humidified atmosphere (5.5% CO_2) at 37°C. For each experiment, the cells underwent two passages, one when they were obtained before being frozen, and the other when they were counted and seeded to the various experimental conditions tested. When cells reached 80% confluence, they were starved in 1.5% FBS medium for 7-16 h before starting treatment. Treatments were performed by replacing the medium by osteogenic medium (α-MEM supplemented with 1.5% FBS, 2 mM β-glycerophosphate, 50 μg/mL ascorbic acid) containing the indicated amounts of ATPγ-S, UTP, LiCl, or the vehicle/water pH:7.2 used in control conditions. The control or treatment medium was renewed every 2-3 days.

2.4 | Immunofluorescence

After treatments, cells were washed with PBS and then fixed with methanol for 20 min at −20°C. Next, the samples were washed with PBS (2-3 times) and blocked with BSA (5% in PBS) for 45 min at RT. After another washing, the samples were incubated with the first antibody (anti-β-catenin 1: 300) overnight at 4°C. After washing, the second antibody was added (1: 300) for 1 h at RT. The samples were washed with PBS once and nuclei were stained with propidium iodide for 15 min at RT.

2.5 | Image processing

Immunofluorescence from images obtained was quantified by using ImageJ, as follows. Mean fluorescence intensity was calculated by analyzing 35-40 nuclei from each image of the indicated condition. Only nuclei that were isolated (free from "artefacts" or superposition with other cells) were taken into account, in order to not overestimate the intensity of the staining.

2.6 | Alkaline phosphatase activity (ALP)

The ALP activity of cell was colorimetrically determined using a commercially available kit (Wiener Lab.); this assay uses sodium phenylphosphate as a substrate; ALP in the presence of methyl propanol amine (pH 10) releases phenol. The phenol released is combined with a color generating reagent solution of 4-amino-antipyrine and ferrocyanide and quantified at 520 nm. The ALP activity was measured after the indicated cell treatment. Briefly, cell layers were washed with PBS and then cells were incubated with 50 μl/well of PBS containing 0.2% Triton for 10 min at 37°C. Follow 250 μl

of substrate was added to each sample and it was incubated for 10 min (37 °C) before the addition of 1.25 mL of color reagent and quantification as indicated above. A blank (B) and standard (S) (200 IU/L phenol) were also processed. Optical density of de samples (D) was measured and ALP activity was calculated as follow: ALP (IU/L) = 200 IU/Lx (D−B)/(S−B).

2.7 | Alizarin red staining

Calvarial osteoblasts seeded in 48-well plates were cultured and treated as above for 15, 18, and 22 days. The cells were then fixed with 2% glutaraldehyde in PBS (pH 7.4) at room temperature for 10 min, washed three times with PBS, and then incubated with 2% Alizarin Red (pH 4.2) for 30 min at 37°C. The cells were washed thoroughly with deionized water. After Alizarin Red staining, the samples were incubated with 0.1 M NaOH and O.D. (Absorbance) were measured by spectrophotometer at 548 nm.

2.8 | Neutral red staining

After treatments, cells were washed with PBS 1X and stained with Neutral red for 2-3 h, at 37°C. Dye excess was removed with PBS 1X. Finally, the dye incorporated to cells was extracted with destain solution (50% ethanol 96%, 49% deionized water, 1% glacial acetic acid) and quantified, at 540 nm, in a spectrophotometer with plate reader.

2.9 | Statistical analysis

Statistical significance of data was evaluated using Student's *t*-test and probability values below 0.05 ($P < 0.05$) were considered significant. Quantitative data are expressed as means ± standard deviation (SD) from the indicated set of experiments.

3 | RESULTS

3.1 | ATPγ-S stimulates the accumulation of β-catenin in cytoplasm and its translocation to nucleus in rat primary calvarial cells

In our previous work, we demonstrated that ATP stimulates the proliferation and differentiation of rat primary calvarial cells involving the PI3K/AKT pathway.[15] As AKT was shown to inhibit GSK3, generating accumulation, and stabilization of β-catenin,[42,43] we evaluated the effect of ATP on location and expression of β-catenin by using immunofluorescence, as indicated in section 2. It was observed that cell treatment with 10 or 100 μM ATPγ-S, for 96 h increased the cytoplasmic and nuclear levels of β-catenin respect to control. In addition, quantification of nuclear β-catenin shown to be significantly increased in cell treated with 100 μM ATPγ-S respect to control, whereas 10 μM ATPγ-S cell treatment showed a tendency to increase, which was not significant but should not be underestimated. Similar effect was observed after cell treatment with the GSK3 inhibitor LiCl (10 mM) (Figures 1A and 1B). These data show that ATPγ-S increases the cytoplasmic levels of β-catenin and its translocation to cell nucleus. Temporal correlation with LiCl effects, also suggest the involvement of GSK3/β-catenin signaling pathway in the action of ATP on rat calvarial cells.

3.2 | Treatments with ATPγ-S or the GSK3 inhibitor LiCl stimulate ALP activity of rat calvarial cells

In order to study the involvement of GSK-3 in osteoblasts differentiation of rat calvarial cells, alkaline phosphatase (ALP) activity was measured after different treatments with the GSK-3 inhibitor LiCl. Cells were cultured in osteogenic medium in the presence or absence of 4-10 mM LiCl for 4 and 7 days. Then ALP activity was measured as indicated in section 2. As the Figure 2 show, 4-10 mM LiCl treatments significantly stimulates ALP activity respect control at 4 and 7 days, suggesting that inhibition of GSK-3 mediates osteoblasts differentiation of rat calvarial cells.

In order to compare the effect of LiCl with the ATP on ALP activity, cells were cultured in osteogenic medium in the presence or absence of 10 mM LiCl or 10 μM ATPγ-S for 4-10 days. Then ALP activity was measured as indicated in section 2. We observe that, at 4 and 7 day of treatment, LiCl but not ATPγ-S stimulates ALP in a significant manner respect control. However, at 10 day of treatment, the ALP activity was significantly increased by ATPγ-S and decreased by LiCl respect to control (Figure 3). These data suggest that the effect of ATPγ-S was less potent but more persistent than of LiCl in stimulating ALP activity in calvarial cells. In addition, this result suggests that strong inhibition of GSK3 activity by LiCl could be negatively affects osteoblasts differentiation at longer times.

To assess whether the LiCl action has a synergistic effect regarding purinergic stimulation, ALP activity was determined after treatment of the cells with 10 mM LiCl together with 10 or 100 μM UTP, an agonist of P2Y2 and P2Y4 (in rat) receptors. As the Figure 4 shows, 10 mM LiCl stimulated the ALP activity, at 4 and 7 days of treatment, but UTP did not. On the other hand, the joint treatment showed no interaction between the two agents (Figure 4).

3.3 | Effect of ATPγ-S or LiCl treatment on rat calvarial cell cultures mineralization

Calvarial osteoblasts reach a mature stage after long incubation times (>15 day) in osteogenic medium. Mineralization nodules can be visualized around day 15-20, or even

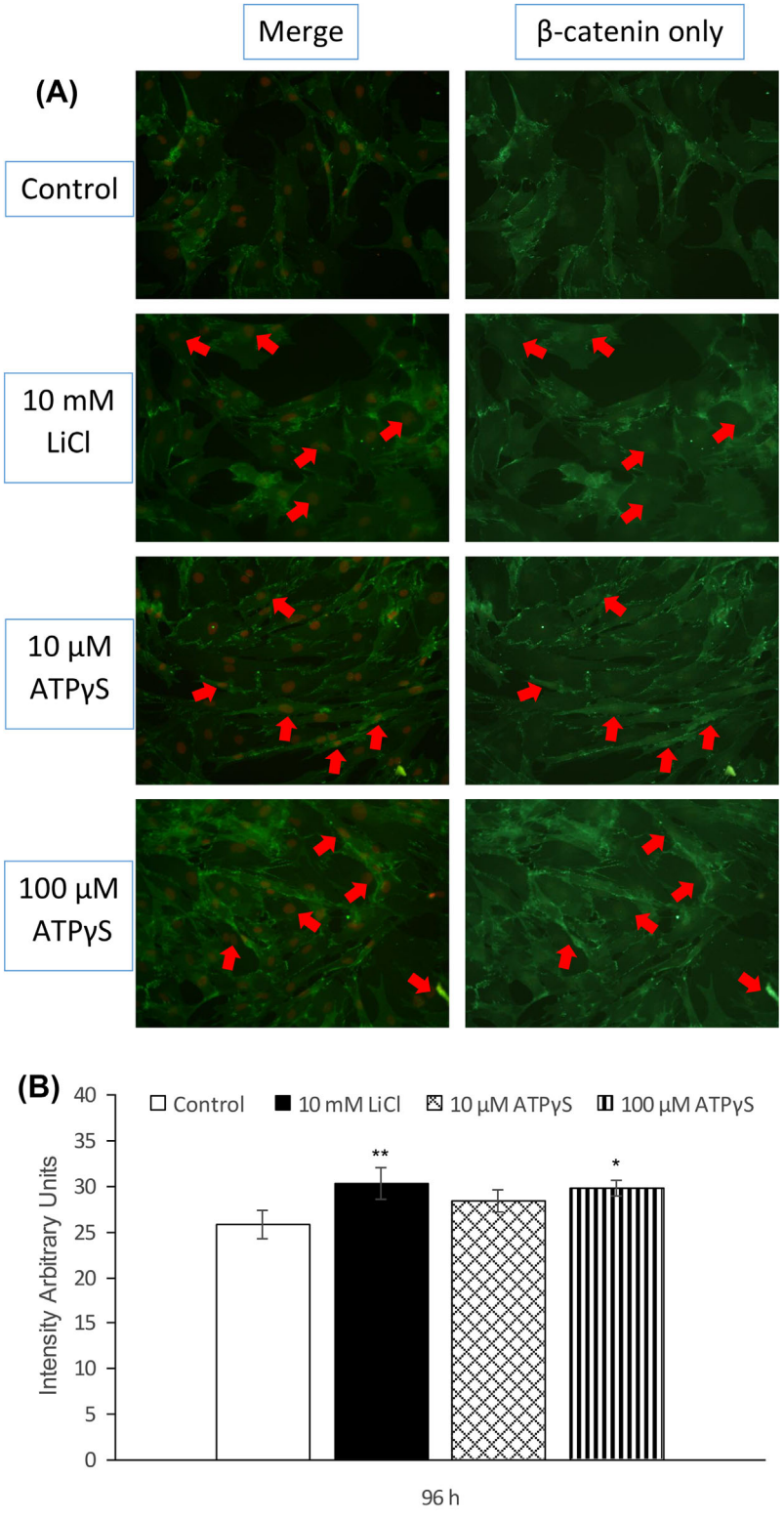

FIGURE 1 ATPγ-S or LiCl treatment stimulates the accumulation of β-catenin in cytoplasm and its translocation to nucleus in rat primary calvarial cells. Cells were incubated in osteogenic medium in the presence or absence of 10-100 µM ATPγ-S or 10 mM LiCl for 96 h. Then, detection of β-catenin and its nuclear quantification were performed as indicated in section 2. Images representative are shown in (A) and (B), nuclear immunofluorescence quantification by ImageJ program analysis was obtained; each value is the average ± SD of 35-40 nuclei per condition from two independent experiments. $*P < 0.05$; $**P < 0.025$ versus control

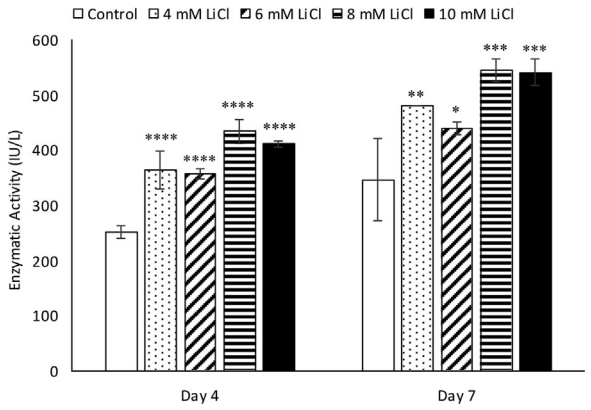

FIGURE 2 Effect of LiCl treatment on ALP activity in neonatal rat calvarial cells. Cells were incubated in osteogenic medium the presence or absence of 4-10 mM LiCl for 4 and 7 days. Then, quantification of ALP activity was performed as indicated in section 2. Each value is the average ± SD of three independent experiments performed in triplicate. * $P < 0.05$; ** $P < 0.025$; *** $P < 0.005$; **** $P < 0.001$ versus control

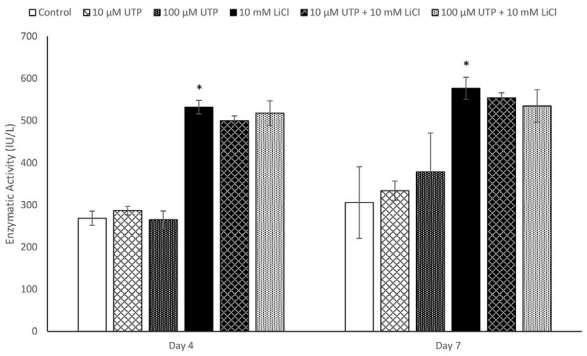

FIGURE 4 Effect of treatment with LiCl and UTP on ALP activity in neonatal rat calvarial cells. Cells were incubated in osteogenic medium in the presence or absence of the indicated concentrations of UTP, LiCl or both for 4 and 7 days. Then, quantification of ALP activity was performed as indicated in section 2. Each value is the average ± SD of two independent experiments performed in triplicate. *$P < 0.025$ versus control

later in such conditions.[20,21] In order to study if inhibition of GSK3 by LiCl is involved in osteoblasts culture mineralization, the effect of cell treatment with 10 mM LiCl on extracellular matrix mineralization after long incubation times was assessed and compared with the action of 10 μM ATPγ-S. This was performed by determining the calcium deposits on cell cultures using the Alizarin Red organic dye, as described in section 2. Cells were treated with 10 mM LiCl or 10 μM ATPγ-S in osteogenic medium for 15, 18, and 22 days.

Cells grown in the presence of 10 mM LiCl showed a statistically significant reduction in calcium deposits at all times studied, respect to control. While, cell treatment with 10 μM ATPγ-S induced a significant increase in mineralization at 15 and 18 days with respect to control (Figure 5). This result suggests that long time exposition of cell to 10 mM LiCl did not favor osteoblasts mineralization. We think that LiCl can affect cell growth or viability. In order to view that, cell growth and viability was assessed after cell exposition at different concentration of LiCl for long time.

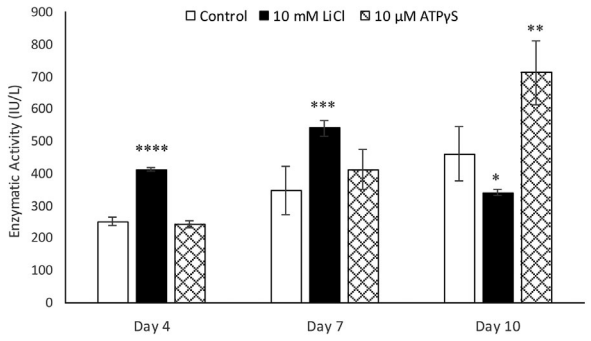

FIGURE 3 Effect of treatment with LiCl or ATP on ALP activity in neonatal rat calvarial cells. Cells were incubated in osteogenic medium in the presence or absence of 10 μM ATPγ-S or 10 mM LiCl for 4, 7, and 10 days. Then, quantification of ALP activity was performed as indicated in section 2. Each value is the average ± SD of three independent experiments performed in triplicate. * $P < 0.05$; ** $P < 0.025$; *** $P < 0.005$; **** $P < 0.001$ versus control

3.4 | Effect of LiCl treatment on rat calvarial cell viability

In order to evaluate cell viability, we used the Neutral Red staining. It is extensively employed as a convenient and rapid assay for measuring cell viability. It is a well known quantitative colorimetric method based on the uptake of the weakly cationic dye Neutral Red which enters into the cell by diffusion through the cell membrane. Then, the dye accumulates in the lysosomes of living cells.[49] The cultures were incubated with different concentrations of LiCl (4-10 mM), during 4, 7, and 10 days, and then processed as described in section 2. As seen in Figure 6, LiCl treatment strongly diminish cell viability at day 10 with all concentration studied, being the more potent effect for 8 and 10 mM, which reduced cell viability since day 4, and for LiCl 6 mM, this effect was noted from day 7.

4 | DISCUSSION

Neonatal rat calvarial cell cultures are a heterogenic cell population majorly containing mesenchymal stem cells, committed osteoprogenitor cells, preosteoblasts, and

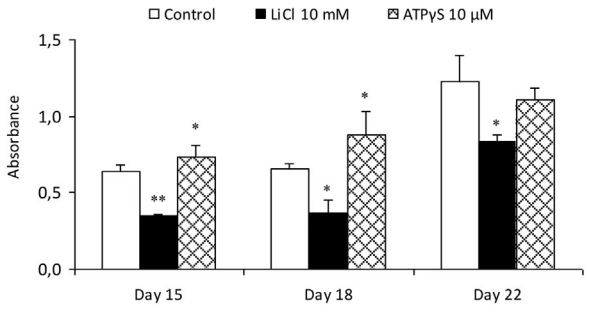

FIGURE 5 Effect of treatment with LiCl or ATP on neonatal rat calvarial cells mineralization. Cells were incubated in osteogenic medium in the presence or absence of 10 μM ATPγ-S or 10 mM LiCl for 15, 18, and 22 days. Then, quantification of mineral matrix deposition after alizarin red staining was performed as indicated in section 2. Each value is the average ± SD of two independent experiments performed in triplicate. * $P < 0.05$; ** $P < 0.01$ vs control

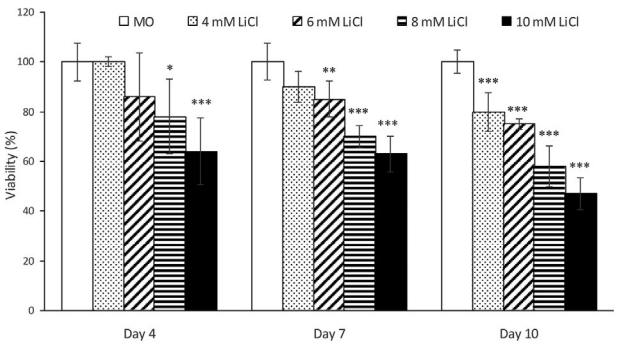

FIGURE 6 Effect of treatment with LiCl on neonatal rat calvarial cells viability. Cells were incubated in osteogenic medium in the presence or absence of 4, 6, 8, or 10 mM LiCl for the indicted times. Then, quantification of cell viability after neutral red staining was performed as indicated in section 2. Each value is the average ± SD of two independent experiments performed in triplicate. *$P < 0.05$; **$P < 0.025$; ***$P < 0.001$ vs control

osteoblasts. Under appropriate conditions, these cell cultures can differentiate into osteoblasts, condrocytes, or adipocytes as it has been described.[50,51] Despite known several osteogenic formulations as well as the importance of each of its constituents for induction of in vitro osteogenic differentiation, this has not yet been fully optimized.[52] Furthermore, the conducing mechanism of osteogenesis has not been totally elucidated.

In the present study, we show for the first time β-catenin accumulation in cytoplasm and its nuclear translocation induced by ATPγ-S in primary cultures of neonatal rat calvarial cells. Our data suggest the involvement of GSK3 inhibition in the purinergic stimulation at early stages of osteogenic cell differentiation. In agreement with this, cell treatment with LiCl increase osteogenic differentiation markers at early stages but decreases the later mineralization process, possibly by impairing cell viability.

It has been shown that treatment of cells with lithium decrease GSK-3 activity as judged by its effect on reducing the phosphorylation of known GSK-3 substrates.[47] Lithium offer an easily boardable way to repress GSK-3 activity and therefore to assess its involvement in various cellular processes.[47,53,54] According with this, temporal correlation between ATPγ-S and LiCl effects on β-catenin cytoplasm accumulation and nuclear translocation observed here, suggest the involvement of GSK-3 in the purinergic action on osteoblasts.

Our data suggests that inhibition of GSK3 is implicated in the increment of ALP activity induced by LiCl in our cell system. In agreement with this, increases in ALP activity induced by LiCl treatment (with some differences in concentration and exposure time) have been observed in murine pre-osteoblasts and mesenchymal cells,[35,55,56] primary bone marrow cell culture of mouse and rat,[57,58] and in human osteoblasts cell lines MG63 and SaOS-2.[59] Moreover, specific GSK3 inhibitors have shown to stimulate ALP activity in ST2 cells[60] and murine mesenchymal cells.[61] In addition, decreased ALP enzymatic activity has been demonstrated in cells transfected with the constitutively active form of GSK3β[62] or after its activation by a lipopolysaccharide.[63] In disagreement with our results, decreases in ALP activity induced by LiCl have been reported in mouse preosteoblastic cells.[64,65] However, in those works, higher concentrations of LiCl (20 and 50 mM) were employed.

Our data suggest that ATPγ-S effect was less potent but more persistent than of LiCl in stimulating ALP activity in calvarial cells. LiCl has shown to inhibit GSK3 by two forms. In a direct manner, lithium inhibits the enzyme by replacing the Mg^{2+} ions at the active site of the protein.[66] In addition, Li^{+} can activate Akt, which results in indirect GSK3 inhibition through phosphorylation mediated by Akt.[48,67] In this regard, ATPγ-S has shown to stimulate PI3K/Akt pathway in rat calvarial cells,[15,21] suggesting that the purinergic agonist could inhibit GSK3 by the indirect mechanism. The lack of synergistic effect between the LiCl action and the purinergic stimulation on the ALP activity observed here, suggests that both signals can converge on a common route or at least not antagonize each other.

Bone mineralization occurs by a set of physicochemical and biochemical processes that facilitate the deposition of hydroxyapatite crystals both along the collagen fibrils in the extracellular matrix,[68] as well as within the lumen of the matrix vesicles derived from osteoblasts.[69] A primary inhibitor of extracellular matrix mineralization is Inorganic pyrophosphate (PPi),[70] produced by the action of ectoenzyme nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1) that catabolizes extracellular ATP to produce PPi and AMP. The role of ATP as a substrate for NPP1 during

the mineralization process has been widely discussed.[71] This work describes the physiological importance of the hydrolysis of ATP to ADP and PPi and the crucial role played by NPP1, tissue-nonspecific alkaline phosphatase, and the phosphatase PHOSPHO1 in the mineralization of normal bone. The authors demonstrate that NPP1 has a role in the generation of PPi at the level of chondrocyte and osteoblast membranes, whereas at the matrix vesicles level, NPP1 does not seem to use ATP efficiently. Therefore, ATP can regulate mineralization in two ways, one acting as substrate for ectoenzymes that generate PPi, which inhibits and other stimulating mineralization via activation of purinergic receptors. In this regard, the use of ATPγ-S, which is very slowly hydrolyzed by phosphatases and most ATPases, suggests that under our working conditions the purinergic signaling is the mechanism predominant and NPP1 action would not be relevant, although we cannot completely rule out its participation.

The negative effect of long-term treatment with LiCl on the viability and differentiation of osteoblasts, evidenced by the reduced mineralization of the extracellular matrix, could be due to a strong and sustained inhibition of GSK3 caused by LiCl. In line with this, GSK3 inhibition has been shown to induce apoptosis and reduce cell viability in neuroblastoma cells.[72,73] In addition, lithium has been shown to facilitate apoptotic signaling and induce autophagy.[74] Interestingly, two enzymes, pyruvate kinase, and inositol monophosphatase A1, both regulating key metabolic events in the eukaryotic cells have been shown directly inhibited by lithium ions.[75,76] On the other hand, it has been demonstrated that lithium can alter cell permeability to monovalent cations[77] and inhibit Na^+/H^+ exchangers,[78,79] proteins that mediates acidification of intracellular vesicles. Based on these evidences, we cannot rule out the involvement of any of these Li^+ actions in its detrimental effect on osteoblast survival and mineralization observed here. Notably, LiCl concentrations that impaired cell viability (8-10 mM) enhanced ALP activity, even more than lower concentrations (4-6 mM) which resulted innocuous to cells, at the same periods. This could mean that the powerful action of LiCl on the stimulation of ALP activity may be related to its effect on cell survival.

Although the joint treatment of LiCl with the purinergic agonist UTP did not show a synergic effect on ALP activity at the concentrations and times studied here, we think that the inclusion of LiCl in the first steps of osteoblasts differentiation can improve the process but this will be aim of future investigations. Of note, LiCl concentrations employed here, appropriate to achieve the greatest magnitude of GSK-3 inhibition,[47] are much higher than those used in human therapy (0.6-1, 2 mM).[80] The effect of the LiCl inclusion, at concentrations within the range used in human therapy, on the differentiation of osteoblasts in the early stages will be the aim of future studies.

In conclusion, our results suggest that ATPγ-S stimulates beta-catenin accumulation in cytoplasm and its translocation to nucleus mediated by GSK3 inhibition in rat calvarial cells. Of relevance, mechanical stretching of human pre-osteoblasts, which is also associated with nucleotide release,[2,4,7] has been shown to induce a biphasic alteration of nuclear localization of β-catenin prior to the initiation of mineralization.[81] In addition, although LiCl appeared to improve osteoblasts differentiation in early phases, it reduced cell viability and calcium deposition in later stages.

This knowing may be of potential application in the development of new study methods in tissue engineering of mineralized bone in vitro, could offer an alternative approach to osseous regeneration by shortening the obtaining time of mature osteoblasts capable of synthesizing new bone in vitro, and also to address commitment of progenitor cells into osteoblasts lineage for implant in bone tissue reconstitution in vivo.

A harmonious combination of musical notes makes a melody sound good. Similarly, an appropriate cellular response depends on the concerted action of different signal transduction pathways, in which purinergic signaling pathways are involved. The future challenge will be to understand how to get the tone when this harmony is lost.

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CONFLICTS OF INTEREST

Authors declare that they do not present conflicts of interest.

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