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Article

Deciphering the Cellular Effects of Strontium Chloride and Potassium Carbonate on Induced Pluripotent Stem Cells and Their Derivative Cardiomyocytes

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Abstract

Background/Objectives: Toothpaste ingredients such as strontium chloride ($SrCl_2$) and potassium carbonate (K_2CO_3) are recognized for their desensitizing and remineralizing effects but may be absorbed through the oral mucosa. Their potential cytotoxic and cardiotoxic properties, however, remain inadequately characterized. Here, we investigated the effects of $SrCl_2$ and K_2CO_3 on mouse-induced pluripotent stem cells (iPSCs) and iPSC-derived cardiomyocytes (iPSC-CMs). **Methods:** Cells were exposed to varying concentrations of each compound for up to 72 h. Real-time cell analysis (xCELLigence RTCA Cardio system) was used to assess proliferation, and flow cytometry was used to evaluate cell viability. Functional properties of iPSC-CMs were examined using multi-electrode array (MEA) recordings and the xCELLigence based impedance measurements. Cardiac marker expression was examined via immunofluorescence and quantitative RT-PCR. **Results:** Both $SrCl_2$ and K_2CO_3 affected iPSCs proliferation and reduced viability in a dose- and time-dependent manner, accompanied by altered embryoid body (EB) morphology and increased cell death. In iPSC-CMs, both compounds downregulated keys cardiac genes and disrupted spontaneous beating activity, with effects intensifying at the higher concentrations. **Conclusions:** These results demonstrate that $SrCl_2$ and K_2CO_3 induced dose-dependent cytotoxic and arrhythmogenic effects on iPSCs and iPSC-CMs. At elevated concentrations, these compounds impair iPSC-CMs function and may pose safety concerns upon chronic exposure. Further mechanistic and long-term in vivo studies are warranted to assess their potential cardiotoxic risk in consumer oral care products.

Keywords: dental; strontium chloride; potassium carbonate; cytotoxicity; pluripotent stem cells; proliferation

1. Introduction

Toothpaste is an essential component of daily oral hygiene and is widely used across all age groups. Although not intended for ingestion, many of its components can remain in the oral cavity after brushing. The oral mucosa, due to its high permeability and vascularization, enables rapid

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absorption of substances [1]. Consequently, ingredients in toothpaste may exert effects beyond the oral cavity, potentially influencing sensitive organs such as the heart. Modern toothpaste formulations contain a range of active and inactive ingredients, such as abrasives, humectants, surfactants, flavoring agents, and preservatives, along with pharmacologically active components targeting specific oral health issues such as tooth sensitivity [2,3]. $SrCl_2$ is a commonly used desensitizing agent that acts by occluding dentinal tubules, thereby reducing fluid movement and neural excitation [4,5]. Belong dentistry, $SrCl_2$ has been shown to alleviate skin irritation [6,7], and to promote periodontal cell proliferation and mineralization at low concentrations [8,9]. Despite these beneficial effects, concerns have been raised about its safety, particularly with regard to cardiovascular health. Epidemiological studies have linked strontium-based medications to an increased risk of cardiovascular events [10], although the exact underlying mechanisms remain unclear. Importantly, the direct effects of $SrCl_2$ on iPSCs and iPSC-CMs have not been investigated.

Potassium carbonate (K_2CO_3) is another ingredient occasionally included in natural or mild toothpaste formulations, though its specific role in these products remain poorly defined. K_2CO_3 is widely used in industrial and pharmaceutical contexts [11–13]. However, due to the its alkaline nature, K_2CO_3 may act as a mucosal irritant at high concentrations, suggesting possible biological reactivity [13]. Its effects on excitable cells, including CMs, are largely unknown, and data on its cardiac safety are currently lacking.

To address these knowledge gaps, the present study investigated the cytotoxic and cardiotoxic effects of $SrCl_2$ and K_2CO_3 using a murine iPSC-based in vitro model. Cell proliferation and viability were assessed in undifferentiated iPSCs, while functional and molecular endpoints were evaluated in iPSC-CMs. A combination of advanced and innovative analytical tools and techniques, including real-time cell analysis (xCELLigence RTCA Cardio system), multi-electrode array (MEA) recordings, fluorescence-activated Cell Sorting (FACS), immunocytochemistry, and quantitative RT-PCR, was employed to characterize both acute and dose-dependent effects. By integrating multiple complementary assays within a physiologically relevant system, this study provides novel insights into the toxicological and functional profiles of $SrCl_2$ and K_2CO_3. The findings may help refine safety assessments for dental care products and guide future research on potential side effects of topically applied compounds.

2. Results

2.1. Mouse iPSC Maintenance and Cardiac Differentiation

Cell culture protocol is depicted in Figure 1A. Briefly, murine iPSCs (AT25) were stably maintained on a neomycin-resistant feeder layer, exhibiting typical colony morphology during expansion, with passaging occurring every 2 days. Differentiation was initiated through embryoid body (EB) formation (Figure 1B) in the presence or absence of various concentrations of $SrCl_2$ and K_2CO_3. After two days of suspension culture, EBs were successfully formed. These EBs were then exposed to the respective compound treatments and monitored throughout the subsequent period until final analysis. At days 5 and 10 post differentiation, no significant changes in EBs size were observed across all concentrations of $SrCl_2$ tested, as compared to control conditions (Figure 1C). In contrast, exposure to K_2CO_3 at low concentration (0.6 mM) resulted in significant increase in EB size. However, at high concentration (3.2 mM), K_2CO_3 induction in EB size, decreasing it to approximately half of the size observed from those in control condition (Figure 1D. Spontaneous beating of the EBs was generally first observed between day 6 and 8 post-differentiation. Simultaneously, eGFP expression under the myosin heavy chain promoter was detected in beating areas, indicating cardiomyogenic lineage commitment. Immunofluorescence analysis of EB cluster, revealed positive staining for α-actinin, a cardiac-specific marker. As shown in Figure 2E, expression of α-actinin was less intense in EBs treated with high concentrations of K_2CO_3 compared to controls, suggesting that these treatments may at least in part affect cardiomyogenesis.

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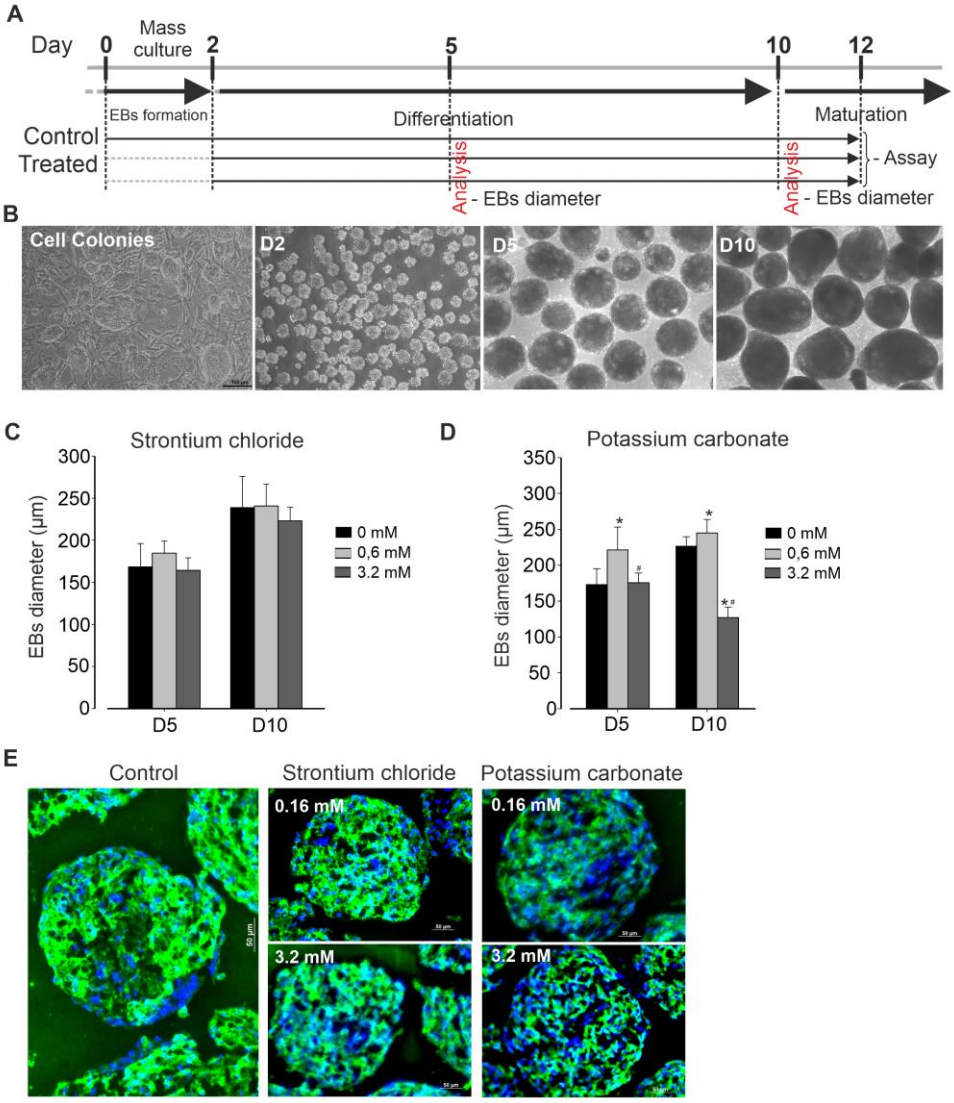

Figure 1. Differentiating murine induced pluripotent stem cells (iPSCs) into cardiomyocytes (CMs). (**A**) Schematic representation of the experimental protocol. (**B**) Representative microscopic images showing cell colonies and embryo bodies (EB) at different stages of differentiation. (**C, D**) Bar graphs illustrating the statistical analysis of EB diameters under Strontium Chloride (B) and Potassium Carbonate (C) treatments compared to control conditions, based on triplicate experiments. Each bar represents the mean EB diameter, with error bars indicating each condition's standard deviation (SD). The graphs compare EB size across various differentiation stages, highlighting the impact of SC treatment on EB growth and development. **E**) Staining of cardiac α-actinin (green) on non-dissociated clusters of beating day 14 EBs differentiated in the absence or presence of the indicated compound. Nuclei were counterstained with Hoechst. *p<0.05 vs. control, #p<0.05 vs. previous concentration.

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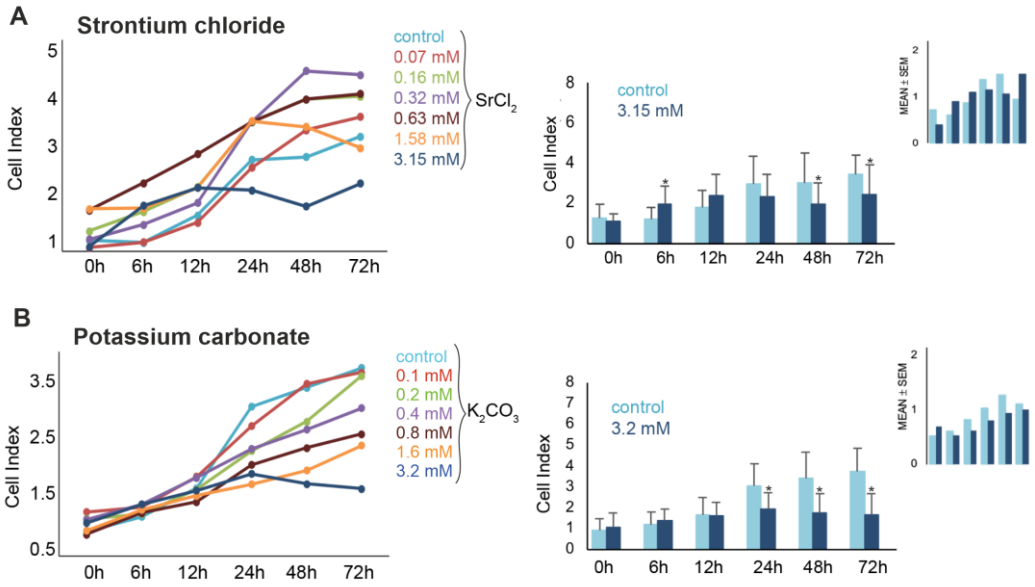

Figure 2. Dose- and time-dependent effects of $SrCl_2$ and K_2CO_3 on iPSC proliferation measured via xCELLigence real-time cell analysis. **(A)** $SrCl_2$ exposure induced a variable response in murine iSPCs. Lower concentrations (≤ 0.68 mM) showed a moderate increase in cell index (CI) over time, whereas higher concentrations (1.58 – 3.15 mM) caused a delayed suppression of CI, particularly after 48 – 72h ($p<0.05$). **(B)** K_2CO_3 exposure led to a more pronounced and concentration-dependent inhibition of proliferation. CI declined significantly at ≥ 1.6 mM starting at 24 h ($p<0.05$). Right bar graphs show mean CI values ± SD at 3.15 mM vs. control over time (n=3). Small insets (top right) illustrate inter-experimental variability for 3.15 mM vs. control, shown as CI range with error bars. These highlight that the observed effects, especially for $SrCl_2$, were subject to biological variation across independent experiments.

2.2. $SrCl_2$ and K_2CO_3 Reduce iPSCs Proliferation

The effect of both compounds $SrCl_2$ and K_2CO_3 on the viability of murine pluripotent stem cells were further examined over a period of 72 h. Real-Time impedance monitoring revealed a biphasic response to $SrCl_2$ exposure (Figure 2A). At lower concentrations (0.07 - 0.63 mM), a transient increase in CI was observed during the first 6 - 12 h. However, higher concentrations (≥ 1.58 mM) induced a progressive decline in CI, with a significant reduction at 3.15 mM (down to ~46% of control at 72 h, $p<0.05$). Intermediate concentrations (0.16 - 0.63 mM) occasionally exhibited proliferative effects depending on the replicate, suggesting inter-experimental variability. Microscopic analysis confirmed reduced cell density and apoptotic morphology after 3.15 mM treatment. FACS further validated this effect, showing a dose-dependent increase in PI-positive cells from 2.4 % (control) to 12.0 % at 3.15 mM after 72 h ($p<0.05$).

Exposure to K_2CO_3 resulted in a reproducible, concentration- and time-dependent decrease in cell viability (Figure 2B). Real-time impedance analysis showed significant CI reductions at 1.6 mM and 3.2 mM K_2CO_3, beginning as early as 24 h and persisting through 72 h. At 3.2 mM, CI values dropped below 50% of the control by 48-72 h ($p<0.05$), indicating pronounced cytotoxicity. In contrast to $SrCl_2$, no significant proliferative responses were observed at any concentration of K_2CO_3 before 24 h. Microscopy examination of cells treated with 3.2 mM for 72 h revealed reduced cell density, detachment, and morphological signs of cell damage (data not shown). These findings were further corroborated by FACS data (Figure 3A-3C), which revealed significant increase in PI-positive cells to 12.7% at 24 h, and 16.2% at 72 h (both $p<0.05$ vs. control) (Figure 3B and 3C, confirming the cytotoxic effect. Taken together, these results demonstrate that exposure to K_2CO_3 consistently induced dose-dependent iPSCs death, with high concentrations leading to substantial loss of viability.

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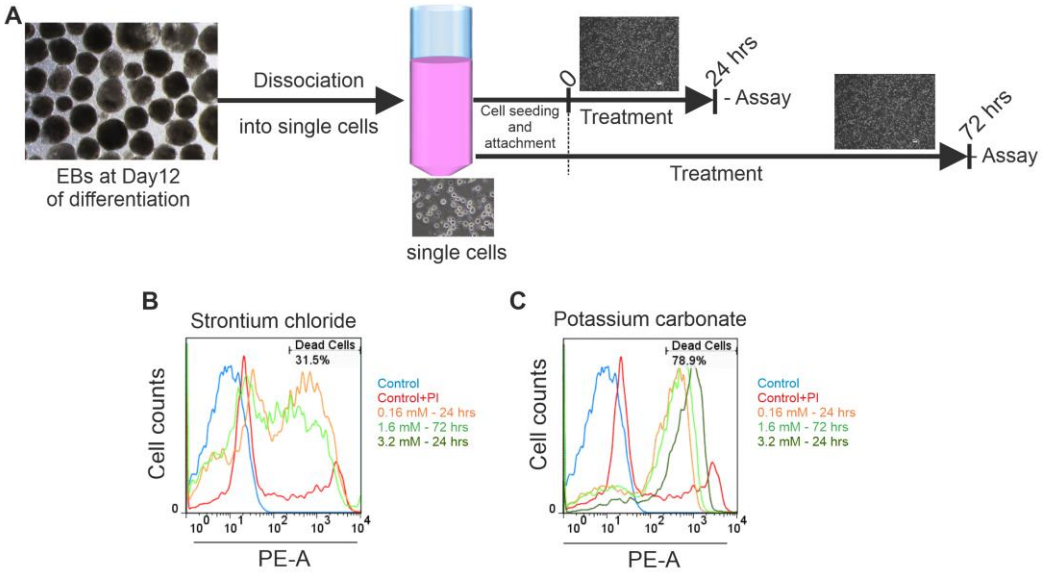

Figure 3. Assessment of Cell Viability and Structural Integrity in iPSC-CMs Following $SrCl_2$ and K_2CO_3 Exposure. **(A)** Diagram of the experimental protocol for flow cytometry analysis illustrating the EBs dissociation, cell seeding, and treatment timeline. **(B-C)** Representative flow cytometry plots showing PI-positive cells within the population treated with varying concentration of $SrCl_2$ (A) and K_2CO_3 (B). The plot demonstrates a significant rightward shift in the treated cells, both in a concentration- and time-dependent manner, compared to untreated cells, indicating cell damage. **(**PI, propidium iodide.

2.3. $SrCl_2$ and K_2CO_3 Modulate Beating Activity of iPSC-CMs Clusters Assessed by MEA

To further investigate the effects of $SrCl_2$ and K_2CO_3 on spontaneously beating clusters of cardiomyocytes, electrophysiological recordings were conducted using the MEA system. Figure 4A presents a representative image of day 12 embryoid bodies (EBs) plated onto an MEA chamber and recorded after attachment. As illustrated in Figures 4B and 4C, low concentrations of both compounds had minimal impact on field potential amplitude and beating frequency (Figure 4B-4G). Specifically, $SrCl_2$ at concentration range from 0.2 to 0.6 mM produced stable field potential traces without significant alterations in spike amplitude or frequency. However, at concentrations above 1.6 mM, $SrCl_2$ induced noticeable changes in both parameters. In contrast, K_2CO_3 caused more pronounced and dose-dependent disruptions. At 3.2 mM, spike morphology became less defined, and field potential amplitude was reduced compared to the control. These effects were supported by quantitative analysis, which showed a consistent trend toward decreased spike amplitude at 1.6 mM and 3.2 mM for both compounds, although variations in beating frequency were less consistent. Taken together, both $SrCl_2$ and K_2CO_3 exhibited minimal functional effects on iPSC-CMs at low concentrations, while higher concentrations led to significant electrophysiological disturbances, detectable through MEA-based field potential analysis.

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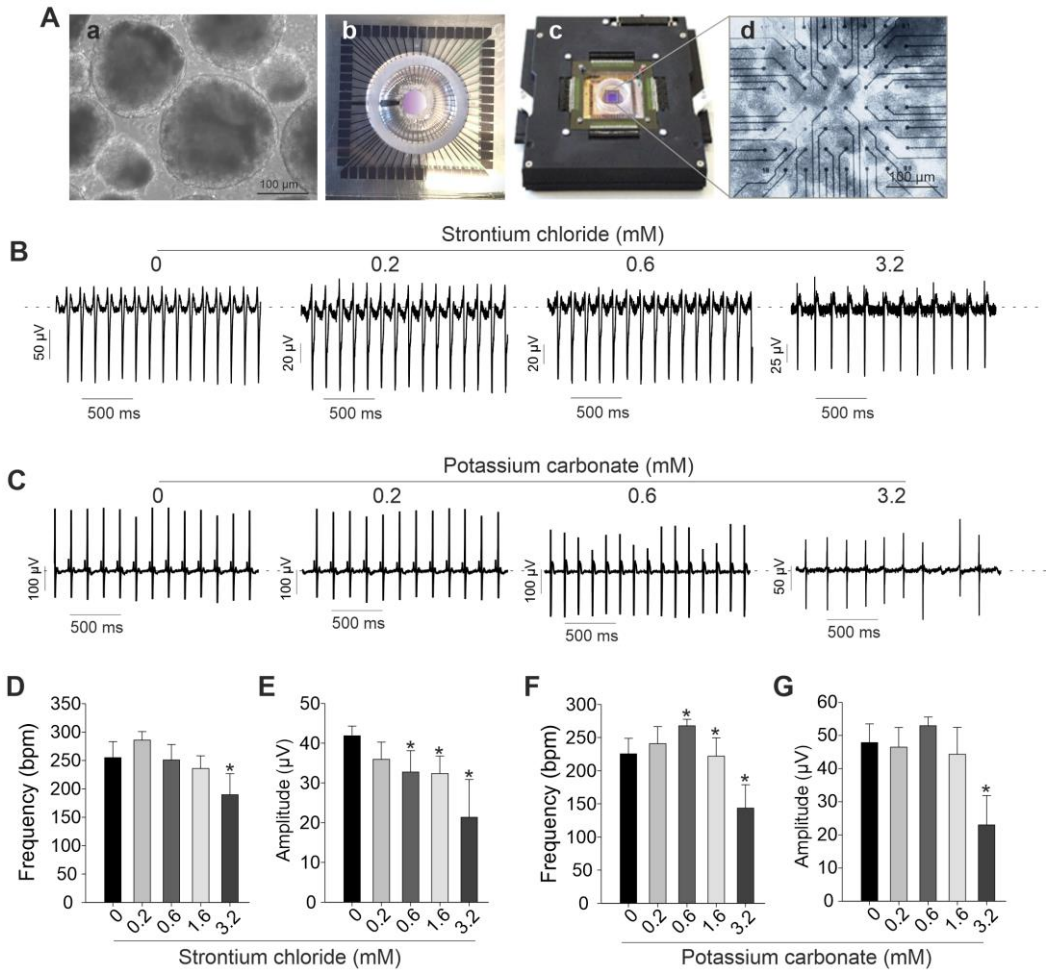

Figure 4. Functional effects of $SrCl_2$ and K_2CO_3 on iPSC-CMs beating clusters were assessed by MEA. **(A)** The setup and cell model used for MEA analysis. **(a)** Brightfield image of EBs at day 12 post-differentiation; **(b-d)** MEA chip and amplifier system used for field potential recordings of iPSC-CMs seeded on MEA electrodes. **(B, C)** Representative FP traces of iPSC-CMs treated with increasing concentrations of $SrCl_2$ **(B)** or K_2CO_3 **(C)**. Traces demonstrate effects on frequency and FP amplitude across 0 – 3.2 mM concentrations. **(D, E)** Quantification of beating frequency **(D)** and FP amplitude **(E)** in $SrCl_2$-treated iPSC-CMs. While beating frequency was not significantly altered at lower doses, a significant reduction was observed at 3.2 mM. Field potential (FP) amplitude decreased significantly at concentrations ≥ 0.2 mM. **(F, G)** Quantifying beating frequency (F) and FP amplitude (G) in K_2CO_3-treated iPSC-CMs. Low doses (0.2 - 0.6 mM) increased frequency significantly, while 3.2 mM caused a strong decrease. FP amplitude was significantly reduced at 3.2 mM. All values are presented as mean ± SEM (n=3-4), $p<0.05$ vs. control (0 mM)..

2.4. Impedance-Based Analysis Reveals $SrCl_2$ and K_2CO_3 Disrupt Coordinated Beating in iPSC-CMs

To distinguish the effects of $SrCl_2$ and K_2CO_3 on the beating patterns of 2D cardiac cell cultures, real-time impedance analysis was performed using the xCELLigence RTCA system. This platform enables continuous, label-free monitoring of cellular dynamics, capturing changes in cell adhesion, morphology, and contractile activity. By analyzing impedance signals, alterations in beating frequency and potential arrhythmic events induced by chemical exposure or genetic modifications can be effectively assessed. As revealed exposure of iPSC-CMs to $SrCl_2$ and K_2CO_3 induced changes in the CI and beating pattern in a concentration-dependent manner (Figure 5A). Both compounds do not appear to impede cell attachment or monolayer formation, as indicated by the normal progression of the CI. At the high concentration of 3.2 mM, prolonged exposure led to a progressive decline in CI over 72 h for both compounds, with a more pronounced reduction observed for K_2CO_3. Lower concentrations (0.6 - 1.6 mM) had minimal effects on CI values compared to the controls. Beating

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profiles recorded via the RTCA system revealed that $SrCl_2$ preserved rhythmic contractions at 0.6 and 1.6 mM. At 3.2 mM, contractions remained regular but showed reduced amplitude and frequency (Figure 5B, left). In contrast, K_2CO_3 at 3.2 mM induced irregular and low-amplitude beating (Figure 5B, right), indicating impaired excitation-contraction coupling. Quantification of beating frequency confirmed these observations (Figure 5C). SrCl2 caused a significant reduction only at 3.2 mM ($p<0.05$), while K_2CO_3 induced a dose-dependent decrease starting at 1.6 mM, with frequencies dropping below 100 bpm at 3.2 mM ($p<0.05$). These results demonstrate dose -dependent functional impairment in iPSC-CMs, with K_2CO_3 causing more severe and earlier effects than $SrCl_2$.

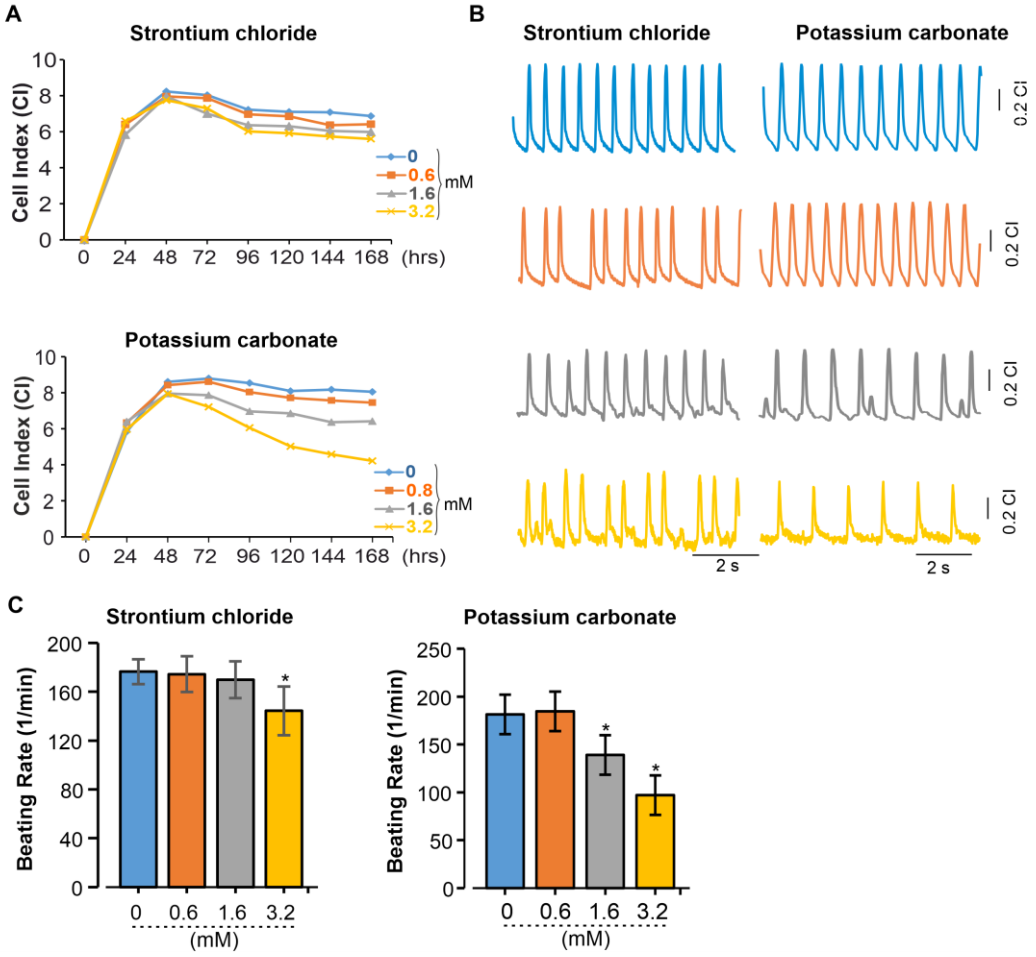

Figure 5. Functional effects of $SrCl_2$ and K_2CO_3 on iPSC-CM were assessed using xCELLigence RTCA Cardio System. (**A**) Representative graph from one of three independent experiments showing the effects of $SrCl_2$ and K_2CO_3 on miPSC-CMs. The CI was monitored in real time for about 196 h after the addition of 0 mM (control) or various concentrations of $SrCl_2$ and K_2CO_3. (**B**) Representative beating rate pattern from 20 ms recordings of iPSC-CMs exposed to different concentrations of $SrCl_2$ and K_2CO_3. (**C**) Average change in beating rate of iPSC-CMs under different concentrations of $SrCl_2$ and K_2CO_3. Results are reported as the mean ± SEM (n=3 independent experiments). *$p<0.05$ vs. control (0 mM).

2.5. Transcriptional Impact of $SrCl_2$ and K_2CO_3 on iPSC-CMs

To assess the molecular impact of $SrCl_2$ and K_2CO_3 on cardiac gene expression, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed at 24 h and 72 h following exposure to each compound at concentrations of 0.2 mM and 3.2 mM. The analysis focused on a set of genes representing key aspects of cardiac development and function, including early mesodermal differentiation (*Mesp1*), structural sarcomeric proteins (*Myl2, Myh6, Mlc2v*), cardiac ion channels (*Scn5a, Cacna1c, Kcnh2*), and a stress marker (*Nppa*). Expression levels were normalized to *Gapdh* as an internal reference.

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The result revealed that K_2CO_3 induced broad transcriptional suppression in a dose- and time-dependent manner (Figure 6A). *Mesp1* and *Myh6* were markedly downregulated at both time points 24 h and 72 h, with the suppression being more pronounced at higher concentrations (3.2 mM) after 24 h. *Myl2* and *Kcnh2* showed persistent downregulation at all concentration tested. While *Mlc2v* initially showed a decline, its expression recovered at 72 h, indicating some degree of compensation at the lower dose (0.2 mM). *Scn5a* and Cacna1c were also significantly affected, with *Scn5a* showing remarkable downregulation compared to *Cacna1*. In addition, *Nppa*, progressively declined over time, with more pronounced suppression observed at the higher concentration. In contrast, $SrCl_2$ elicited milder and more partially reversible changes in gene expression (Figure 6B). *Mesp1* and *Cacna1c* were transiently downregulated at 24 h in dose dependent-manner, but both recovered by 72 h, indicating a temporary effect. Similarly, *Myl2* was moderately affected at both time points. *Mlc2v* and *Myh6* showed sustained downregulation at tested concentration. *Scn5a* and were only slightly affected, and *Nppa* expression showed partial recovery after 72 h.

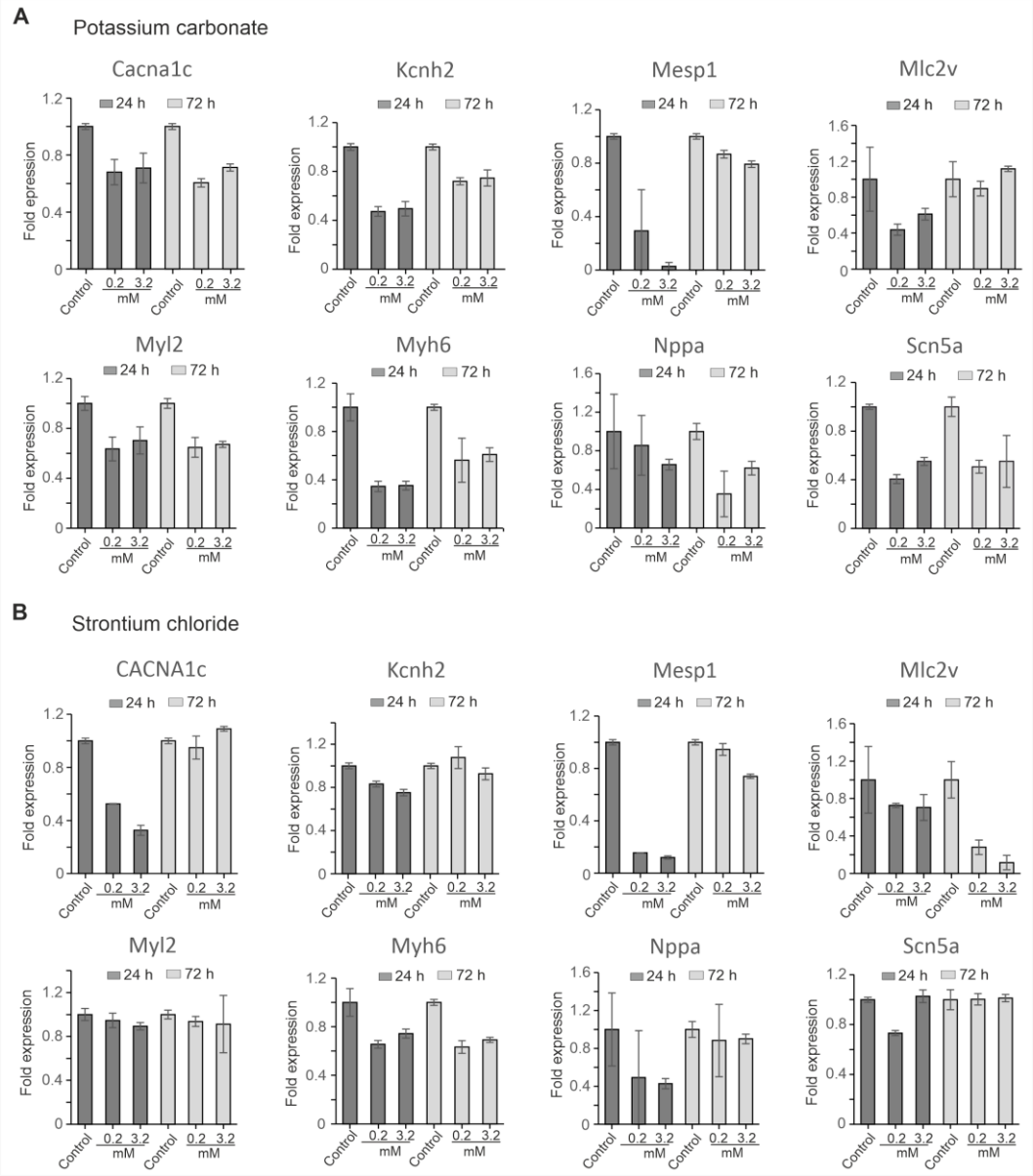

Figure 6. qRT-PCR analysis of cardiac gene expression in iPSC-CMs treated with $SrCl_2$ and K_2CO_3. **(A)** Gene expression levels after treatment with 0.2 mM and 3.2 mM K_2CO_3 at 24 h and 72 h relative to the untreated control. Expression of cardiac progenitor marker (*Mesp1*), structural genes (*Myl2*, *Myh6*, *Mlc2v*), ion channel genes (*Scn5a, Cacna1c, Kcnh2*), and stress marker (*Nppa*) was assessed. K_2CO_3 exposure caused widespread and

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persistent downregulation across several markers, particularly *Mesp1*, *Myl2*, *Myh6*, and *Kcnh2*. **(B)** Gene expression levels following $SrCl_2$ exposure under identical conditions. $SrCl_2$ treatment induced more transient changes, with several markers (*Mesp1*, *Cacna1c*, *Kcnh2*) showing partial or full recovery by 72 h. Data represent mean ± SEM from independent experiments. Expression was normalized to *Gapdh* and calculated using the ΔΔCt method.

Taken together, K_2CO_3 caused more robust widespread transcriptional repression of multiple categories of genes, including markers of early mesodermal differentiation, structural sarcomeric proteins, ion channels, and stress markers. This indicates that K_2CO_3 has a higher transcriptional toxicity potential than $SrCl_2$ in iPSC-CMs, which exhibited milder and more reversible changes in gene expression. These results suggest that K_2CO_3 may pose a greater risk to cardiac development and function than $SrCl_2$, emphasizing the compound-specific nature of their gene expression profiles and their potential impact on cardiac cells.

Table 1. List of primer used for quantitative RT-PCR. Sequences are shown from 5' to 3' direction.

Gene	Forward Primer	Reverse Primer
Myh6	AACAGGTGATGGCAAGATCC	GCTCAAAGTCAGCACCTTC
Myl2	AAAGAGGCTCCAGGTCCAAT	TCAGCCTTCAGTGACCCTTT
Nppa	GGGGGTAGGATTGACAGGAT	ACACACCACAAGGGCTTAGG
Mesp1	GTCTGCAGCGGGGTGTCGTG	CGGCGGCGTCCAGGTTTCTA
Cacna1c	AAGGCTACCTGGATTGGATCAC	GCCACGTTTTCGGTGTTGAC
Kcnh2	CGTGCTGCCTGAGTACAAGCT	TGTGAAGACAGCCGTGTAGATGA
Scn5a	GAAGAAGCTGGGCTCCAAGA	CATCGAAGGCCTGCTTGGTC
Mlc2v	AAAGAGGCTCCAGGTCCAAT	TCAGCCTTCAGTGACCCTTT
Gapdh	GGTGCTGAGTATGTCGTGGA	CGGAGATGATGACCCTTTTG

3. Discussion

This study investigated the cytotoxic and cardiotoxic properties of two commonly used inorganic compounds $SrCl_2$ and K_2CO_3 using a murine iPSC-derived in vitro model. By combining real-time impedance monitoring, electrophysiological recordings via MEA, FACS, and gene expression analysis, we assessed their impact on both undifferentiated iPSCs and differentiated iPSC-CMS. Our results demonstrate that both compounds interfere with CMs viability and function in a dose- and time-dependent manner, although through distinct and potentially complementary mechanisms.

$SrCl_2$ displayed a variable toxicity profile across assays. While some concentrations transiently stimulated iPSC proliferation, higher doses consistently reduced cell viability and altered contractility. These effects may be related to the chemical similarity between Sr^{2+} and Ca^{2+}, which could allow Sr^{2+} entry via Ca^{2+} channels and interfere with Ca^{2+}-dependent signaling pathways [14,15]. Previous studies revealed that Sr^{2+} can prolong action potentials (AP) and disrupt excitation-contraction coupling [16,17], consistent with the mild arrhythmogenic activity observed in our MEA and RTCA xCELLigence data. Gene expression analysis revealed transient suppression of differentiation and contractile markers, with partial recovered by 72 h, suggesting an adaptive cellular response or incomplete toxicity under the conditions tested. The variability in proliferation responses across replicates is consistent with reported cell type specific effects of $SrCl_2$, which can promote proliferation in some contexts while being cytotoxic in others [18,19].

In contrast, K_2CO_3 exhibited a robust and consistent cytotoxic profile. Exposure to increasing concentrations led in a rapid decrease in cell index, pronounced morphological disruption, and high density cell death. This is likely mediated by membrane depolarization due to excess extracellular K^+ concentration, which may inactivate Na^+ channels [20], and consequent impairment of AP initiation [21,22]. This observation is consistent with our MEA findings demonstrating reduced spike amplitude and contractile silencing. Additionally, the sustained downregulation of some cardiac

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genes, particularly encoding ion channel and structural proteins, suggests a broader disruption of CMs identity and functional integrity.

Both compounds also affected transcriptional programs and stress signaling. Downregulation of *Mesp1*, *Myh6*, and *Kcnh2* observed after compound exposure suggests disruption of cardiac identity, with K_2CO_3 induced more consistent suppression. $SrCl_2$ may interfere with transcription via Ca^{2+}-mimetic signaling pathways, potential implicating NFAT or MEF2 activation [23,25]. Persistent suppression of *Mlc2v* and transient *Nppa* modulation in $SrCl_2$-treated cells may reflect early responses or alterations in ventricular cell and tissue specification. It should also be noted that $SrCl_2$ and K_2CO_3 might exert effects through mechanisms beyond electrophysiology. Since Sr^{2+} has been shown to activate the Ca^{2+}-sensing receptor (CaSR), a G-protein coupled receptor involved in intracellular Ca^{2+} release via IP_3 signaling in bone and thyroid cells [26,27], it remains plausible that a comparable mechanism may occur in CMs. In CMs, such dysregulation could trigger mitochondrial Ca^{2+} overload and apoptosis [28]. Similarly, excess extracellular K^+ may modulate cytokine signaling and pro-apoptotic pathways [29], and carbonate ions of K_2CO_3 may locally alkalinize the medium, potentially introducing secondary stress [28]. Although not directly measured here, ionic imbalances can influence inflammatory pathways [30,31], including TGF-β and TNF-α signaling, which are known to affect CMs survival and function [32,33].

In summary, $SrCl_2$ and K_2CO_3 exhibit distinct, dose-dependent cardiotoxic profiles in iPSC-with SrI_2 induced progressive arrhythmogenic disturbances and K_2CO_3 causing acute electrical silencing. These findings highlight how even simple ionic compounds can exert distinct and complex effects on excitable cells. Although further validation is needed, our observations underscore the importance of functional cardiotoxicity assessments for commonly used substances, including those considered relatively inert.

Limitations

This investigation offers initial insights into the acute effects of $SrCl_2$ and K_2CO_3 on CMs; however, several caveats must be acknowledged. The absence of direct mechanistic investigations, such as assessments of ion channel activity, Ca^{2+} handling, or inflammatory signaling, restricts the mechanistic interpretation of the observed cellular responses. Moreover, the concentrations applied in vitro were not benchmarked against physiologically relevant exposure levels, and corresponding pharmacokinetic or absorption data are unavailable to support translational extrapolation. The focus on short-term exposure (≤72 h) in both MEA recording and gene expression analyses further restricts the translation of these findings to realistic consumer exposure scenarios. Consequently, the present results should be considered hypothesis generating and underscoring the need for follow up studies that incorporate long-term, physiologically based, and mechanistically oriented approaches, including in vivo experiment, to more accurately delineate the cardiotoxic potential of these compounds.

4. Materials and Methods

4.1. Reagents and Compounds

All reagents were purchased from Carl Roth (Germany), unless otherwise stated. Dulbecco´s Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Germany) was used to culture undifferentiated murine iPSCs, while Iscove's Modified Dulbecco's Medium (IMDM) served as the base for cardiac differentiation protocols. Stock solutions of $SrCl_2$ and K_2CO_3 were freshly prepared and diluted in the respective culture media to achieve final concentrations ranging from 0.07-3.2 mM for $SrCl_2$ and 0.1- 3.2 mM for K_2CO_3, immediately prior to application.

4.2. Culture of Murine iPSCs

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The murine αPig-AT25 iPSC line, expressing eGFP and puromycin resistance under control of the αMHC promoter, was maintained on mitotically inactivated mouse embryonic fibroblasts (MEFs). The cells were cultured in DMEM supplemented with 15% fetal bovine serum (FBS), 100 U/mL leukemia inhibitory factor (LIF), 1% non-essential amino acids, 1 mM sodium pyruvate, and 0.1 mM β-mercaptoethanol. Cells were passaged every 48 h using 0.05% trypsin/EDTA.

4.3. Cardiac Differentiation of iPSCs

Cardiomyocytes (CMs) differentiation was induced using the embryoid body (EB) method with slight modifications based on *Fatima* et al. [34]. Briefly, 1×10^6 iPSCs were cultured in suspension in IMDM supplemented with 20% FBS and 1% penicillin/streptomycin under continuous agitation. On day 2, the resulting EBs were plated onto gelatin-coated dishes and maintained under adherent conditions for an additional 7 days. Spontaneous contraction of the EBs was observed between day 7 and 9. Puromycin (8 μg/mL) was added for 72 h to select αMHC-positive CMs, resulting in the enrichment of purified iPSC-CMs.

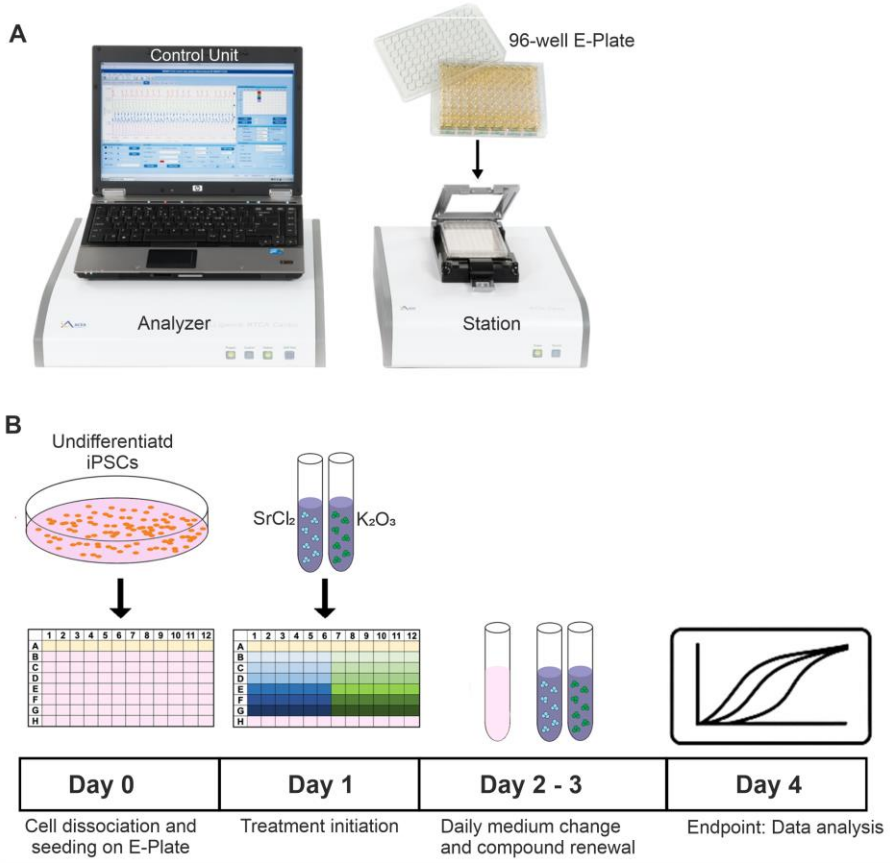

Figure 7. Schematic overview of the experimental setup for xCELLigence-based proliferation monitoring of murine iPSCs. (**A**) The xCELLigence RTCA Cardio Instrument, used for continuous impedance-based analysis of cell proliferation and viability, is used for continuous impedance-based cell proliferation and viability analysis. The system includes an analyzer and control unit, connected to specialized 96-well E-Plates containing interdigitated gold electrodes. (**B**) Timeline summarizing the experimental procedure: after seeding (Day 0), treatment was initiated on Day 1, and cells were exposed to repeated compound application for up to 72 h. Treatments with SrCl2 and K2CO3 were applied at various concentrations. Final analysis was performed on Day4.

4.4. Drug Treatments

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For cytotoxicity and functional evaluations, cells were seeded in appropriate densities on gelatin-coated 96-well E-Plates (xCELLigence RTCA Cardio Instrument, Agilent Technologies, formerly ACEA Biosciences, Figure 7A) or MEA chips and allowed to adhere for 24 h. After adhesion, the cells were then treated with various concentrations of $SrCl_2$ or K_2CO_3. To maintain continuous exposure, the culture medium and compounds were refreshed every 24 h (Figure 7B).

4.5. xCELLigence Real-Time Cell Analysis (RTCA)

The proliferations and cardiotoxicity of iPSCs and iPSC-CMs were assessed using the xCELLigence RTCA Cardio Instrument (Figure 7A). Real-time changes in electrical impedance were recorded as Cell Index (CI), which reflecting cell viability, adherence, and beating behavior (in case of iPSC-CMs) [35]. Data were collected and analyzed using RTCA Cardio Software.

4.6. Field Potential (FP) Recordings Using Microelectrode Arrays (MEA)

Extracellular FP recordings were conducted using a microelectrode array (MEA) recording system equipped with a Multichannel Systems 1060-Inv-BC amplifier and data acquisition system (Multichannel Systems, Reutlingen, Germany). Beating embryoid bodies (EBs) were cultured on MEA dishes that were pre-coated with 0.1%gelatin. The cultures were incubated at 37°C in a 5% CO_2 atmosphere for 1-2 days to allow proper attachment before recording. All recordings were carried out at 37°C to maintain physiological conditions. Various concentrations both $SrCl_2$ and K_2CO_3 were applied to the MEA chambers containing the tissue cultures. After a 3 min baseline recording, different concentrations of both compounds (applied gradually) were introduced, with recordings taken for at least 3 min at each concentration. The data collected from the MEA were analyzed using cardio QT software that was developed in our laboratory with LabView. The analysis focused on the characterization of field potential (FP) frequencies and amplitude.

4.7. Fluorescence-Activated Cell Sorting (FACS)

Cell viability and membrane integrity were assessed via propidium iodide (PI) staining. Following trypsinization, cells were washed with phosphate buffered saline (PBS) and resuspended in PBS containing 1% fetal bovine serum (FBS). To stain death cell, PI (1 μg/mL) was added immediately prior to analysis. Fluorescence was measured using a flow cytometer, and data were analyzed with FlowJo software (version 7). Both iPSCs and iPSC-CMs were assessed for cell death.

4.8. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from iPSC-CMs using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer´s instructions. cDNA synthesis was performed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), using 1 μg of total RNA as starting material. Quantitative RT-PCR was carried out with SYBR Green Master Mix (Bio-Rad, USA) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). The expression levels of cardiac-specific markers, including *Myl2*, *Myh6*, *Mesp1*, *Nppa*, *Mlc2v*, *Scn5a*, *Cacna1c*, and *Kcnh2* were normalized to *Gapdh*. Data analysis was perform using the ΔΔCt method. All reactions were performed in technical triplicate with at least three biological replicates.

4.9. Statistical Analysis

All experiments were performed with at least three technical replicates ($n \geq 3$). Quantitative data are expressed as mean ± SD or as a percentage relative to the control group (set to 100%). Statistical significance was assessed using two-tailed unpaired t-tests, with $p<0.05$ considered significant. For clarity, data were rounded to one or two decimal places in the presentation, while original precision was maintained during statistical analysis.

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Abbreviations

The following abbreviations are used in this manuscript:

K_2CO_3	Potassium carbonate
$SrCl_2$	Strontium chloride
miPSCs	Mouse-induced pluripotent stem cells
iPSC-CMs	iPSC-derived cardiomyocytes
CMs	Cardiomyocytes
MEA	Multi-electrode array
EB	Embryoid body
FACS	Fluorescence-activated Cell Sorting
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
CaSR	Ca2+-sensing receptor

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