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RESEARCH PAPER

Multi-Electrode Array of Sensory Neurons as an *In Vitro* Platform to Identify the Nociceptive Response to Pharmaceutical Buffer Systems of Injectable Biologics

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ABSTRACT

Purpose Pharmaceutical buffer systems, especially for injectable biologics such as monoclonal antibodies, are an important component of successful FDA-approved medications. Clinical studies indicate that buffer components may be contributing factors for increased injection site pain.

Methods To determine the potential nociceptive effects of clinically relevant buffer systems, we developed an *in vitro* multi-electrode array (MEA) based recording system of rodent dorsal root ganglia (DRG) sensory neuron cell culture. This system monitors sensory neuron activity/firing as a surrogate of nociception when challenged with buffer components used in formulating monoclonal antibodies and other injectable biologics.

Results We show that citrate salt and citrate mannitol buffer systems cause an increase in mean firing rate, burst frequency, and burst duration in DRG sensory neurons, unlike histidine or saline buffer systems at the same pH value. Lowering the concentration of citrate leads to a lower firing intensity of DRG sensory neurons.

Conclusion Increased activity/firing of DRG sensory neurons has been suggested as a key feature underlying nociception. Our results support the utility of an *in vitro* MEA assay with cultured DRG sensory neurons to probe the nociceptive potential of clinically relevant buffer components used in injectable biologics.

KEY WORDS buffer system · injection site pain · multi/micro electrode array · nociception · sensory neurons

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ABBREVIATIONS

DRG Dorsal root ganglia
MEA Multielectrode array

INTRODUCTION

Injection site pain (ISP) during subcutaneous administration of therapeutics affects patient compliance (1,2). Fear of pain during injection can manifest as trypanophobia, commonly known as "needle phobia," which may persist throughout life (3–6). The motivation for this research is to provide a tool that focuses on "nociception," a term that describes the peripheral processing of noxious information about the environment, rather than "pain," a term that represents higher-level processing of information by the central nervous system. Pain can be experienced without nociception, and nociception can occur without pain being reported. This makes interpreting human studies difficult. Our method, based on multi-electrode array (MEA) recordings of dorsal root ganglia (DRG)

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neuronal culture, focuses on the nociception component to provide unique insights into the nociceptive behavior of pharmaceutical buffer systems.

Pharmaceutical buffer systems are ubiquitous in stabilizing, optimizing solubility, and regulating pH and tonicity in formulations for delivery of injectable biologics including monoclonal antibodies (7). There are currently about 570 antibody therapeutics at various stages of testing and clinical trials (8). Subcutaneous injection of monoclonal antibodies is challenging due to high protein concentration (9). One of the most common buffer systems used in parenteral formulations is citrate (10). The UK National Health Service reported that over 60% of patients receiving formulations containing citrate report discomfort at the injection site (7,11). Due to the widespread use of buffer systems in injectable medicines, there is a critical need for objective approaches to study the nociception of pharmaceutical buffer formulations *in vitro* to provide higher-throughput screens before clinical trials.

An *in vitro* model to study nociception is using DRG sensory neurons, which relay peripheral nociceptive signals to the spinal cord and then the central nervous system. Hyperexcitability of DRG sensory neuron has been demonstrated as a surrogate for increased nociceptive response (12,13). This makes DRG neurons a relevant model to study nociception. MEA, a relatively high throughput recording system of extracellular field potentials, provides a non-invasive way to continuously monitor neuronal activity and firing patterns of a population of DRG neurons in physiologically relevant conditions as a surrogate of nociception (14). Indeed, we have recently used this system to validate a mathematical model of chemotherapy-induced neuropathy (15). Similar protocols were applied in this work which examines the firing of sensory neurons as an *in vitro* indicator for buffer-associated nociception.

Additionally, studies have shown that sensory neurons carrying mutations from patients with genetic pain syndromes display enhanced firing in MEA recording, which can be reduced by tailored drug therapy, further supporting the utility of MEA recording of sensory neurons as a surrogate to evaluate nociceptive responses and potential interventions (13,16,17). Although this system has been used previously for the pre-clinical investigation of pharmaceutical and chemical agents and disease mechanisms (18–21), it has not been used to probe pharmaceutical buffers systems for injectable biologics. Here, we studied the potential nociceptive profiles of clinical buffer systems being used in formulations for the subcutaneous delivery of injectable biologics.

MATERIALS AND METHODS

Solutions and Reagents

Complete saline solution (CSS) was made from 137 mM NaCl, 5.3 mM KCl, 1 mM $MgCl_2$-$6H_2O$, 25 mM sorbitol, 10 mM HEPES, and 3 mM $CaCl_2$ and equilibrated to pH 7.2. DRG media consisted of Dulbecco's Modified Eagle (DMEM) Medium F12 with 10% bovine serum albumin, glial cell line-derived neurotrophic factor, nerve growth, and antibiotics (penicillin and streptomycin). Buffers were graciously provided by Eli Lilly and Company (Indianapolis, IN, USA). Buffers (pH = 5.7) consisted of the following and named accordingly: 20 mM citrate and 200 mM NaCl ("citrate-salt"), 20 mM citrate and 5% mannitol ("citrate-mannitol"), 10 mM histidine, and 150 mM NaCl ("histidine"), and 150 mM NaCl ("saline"). Low citrate buffers (also pH = 5.7) were made of 10 mM citrate and 150 mM NaCl (named as "low-citrate salt") and 10 mM citrate and 5% mannitol (named as "low-citrate mannitol"). The final recording concentrations (200 μL of buffer in 250 μL of recording media) of the buffers were 8.89 mM citrate and 88.89 mM NaCl ("citrate-salt"), 8.89 mM citrate, and 2.22% mannitol ("citrate-mannitol"), 4.44 mM histidine and 66.67 mM NaCl ("histidine"), 66.67 mM NaCl ("saline"), 4.44 mM citrate and 66.67 mM NaCl ("low-citrate-salt"), and 4.44 mM citrate and 2.22% mannitol ("low-citrate-mannitol"). Osmolarity was determined by Vapor Pressure Osmometer (Wescor, Logan, UT, USA). The osmolarity of the buffers were determined to be 449 ± 4 mOsm for citrate salt, 335 ± 1 mOsm for citrate mannitol, 300 ± 1 mOsm for histidine, and 280 ± 7 mOsm for saline. The osmolarity of the buffer and the media at the end of the recording was 348 ± 1 mOsm for citrate salt, 304 ± 1 mOsm for citrate mannitol, 287 ± 1 mOsm for histidine, and 287 ± 2 mOsm for saline.

Primary Cell Culture

Primary dorsal root ganglia (DRG) from wild-type Sprague-Dawley rat pups (aged 7–17 days) were used for MEA experiments. All animals were maintained and according to the (Purdue) Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. They were group-housed at a constant temperature and humidity on a 12/12 h regular light/dark cycle with ad lib access to food and water in a norovirus-negative facility.

After extraction from the animal, extracted DRG neurons were placed in a complete saline solution (CSS) and treated with collagenase A, collagenase D, and 30 U papain according to previous protocols (Verma *et al.* 2020). The culture was seeded at an average neuron density of 8000 on poly-d-lysine and laminin-coated 12-well MEA plates and incubated for four days at 37°C with 5% CO_2. Media was half-changed (125 μL of DRG media was removed and 125 μL of recording media was added) on the second day to NbActiv4 recording media (BrainBits, Springfield, IL, USA), then recorded on the fourth day.

Since MEA is a population-based analysis, we aimed to minimize variation by having similar seeding density and the number of cells between each group. DRG tissues from several animals were pooled and then the same volume of primary cells was aliquoted to each well to ensure that a similar number of neurons were present in each well. Buffers were randomly added to different wells of each plate (and blinded to the investigator) to further minimize variation.

Micro/Multi-Electrode Array (MEA) Recordings

12-well MEA plates (each well with 64 electrodes) were used for the study. The recording system was a temperature-controlled (37°C) Maestro Pro (Axion BioSystems, Atlanta, GA, USA). The operating volume started at 250 μL of recording media (baseline) and recorded for two minutes (baseline data was collected from the final 30 s of the baseline recording), then 200 μL of testing buffer was added and recorded for two minutes (buffer data was collected from the 30 s after buffer addition), and finally 200 μL of 1.0 μM capsaicin (for a final concentration of 0.3 μM) was added and recorded for two minutes. Capsaicin was used as a positive control to induce neuronal firing to confirm that there were active neurons in the wells around the recording electrodes. If there was no firing (firing was determined as a minimum of two action potential spikes >6 standard deviations above noise) in the baseline, after buffer addition, or after capsaicin addition recordings, then that electrode was removed from the analysis. This was done to eliminate any recording electrodes that did not detect reliable signals.

Manufacturer's software (Axion BioSystems Integrated Studio (AxIS) Navigator 2.0.4 and NeuroMetric Tool 2.4) was used to record and analyze. Active units are defined as an electrode with a minimum of two action potentials spikes (> 6 standard deviations above noise) over the course of the experiment. The average yield of neural recording per well (percent of active electrodes over 64 total electrodes) was 55%. Recordings were from an average of ten different primary DRG cultures from twenty animals and measured on different days. Mean firing rate (Hz), burst firing (≥ 5 spikes over 100 ms), and burst duration were analyzed. The fold change of each electrode was calculated by dividing by baseline of the culture plus one to ensure non-zero denominators (1 + buffer/1 + baseline). Fold changes are reported as well average for each treatment condition.

Statistics

Sample size, defined as one well (average of 35 active electrodes per well), was the following: media $n = 6$ wells (170 total active electrodes), citrate salt $n = 5$ wells (180 active electrodes), citrate mannitol n = 6 wells (199 active electrodes), histidine $n = 7$ wells (269 active electrodes), saline n = 7 wells (206 active electrodes), low citrate salt n = 6 wells (130 active electrodes), low citrate mannitol n = 6 wells (130 active electrodes). Statistical significance was calculated by one-way ANOVA with Bonferroni corrections compared to the media control unless otherwise noted using software GraphPad Prism 8.3.0. Results are presented as mean ± S.E.M. Significance is set at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

RESULTS

Citrate Buffer Systems, but Not Histidine or Saline, Caused a Significant Increase in DRG Neuron Firing

Four buffer systems of citrate salt, citrate mannitol, histidine, and saline were chosen for analysis because of their use in formulation development for high-concentration monoclonal antibody formulations (22,23). Pharmaceutical buffer systems that were studied comprised of the following: 20 mM citrate and 200 mM NaCl ("citrate-salt"), 20 mM citrate and 5% mannitol ("citrate-mannitol"), 10 mM histidine and 150 NaCl ("histidine"), and 150 mM NaCl ("saline"). All buffers were equilibrated to pH 5.7.

Firing patterns were recorded from cultured DRG neurons using MEA to determine the mean firing rate (Hz), burst frequency (Hz), and burst duration (ms), which were chosen as a surrogate to evaluate an *in vitro* nociceptive response based on previous work (16,17,24) (Fig. 1). DRG culture media was used as a control to account for adhesion disturbances due to liquid injection. The qualitative outputs of MEA are heat maps and temporal raster plots. The representative heat maps illustrate action potential events colored by firing frequency with each colored circle within the 8 × 8 electrode array representing active electrodes of firing neurons (Fig. 2). The firing frequency is color-coded with warm colors representing the higher firing frequency and cool colors representing the lower firing frequency.

We found that there is a large increase in firing frequency after the addition of the two citrate buffers and only a slight increase after histidine and saline addition (Fig. 2). The heat-map of a representative well of citrate salt has a medium firing frequency in the baseline (Fig. 2A), and an increase in active neurons to high firing frequency after citrate salt addition (Fig. 2E). A representative well of citrate-mannitol's heat-map shows a couple of active neurons in the before buffer addition (Fig. 2B), and a large increase of active neurons firing at high frequency after buffer addition (Fig. 2F). A representative well of histidine buffer has active neurons of mixed frequency in the baseline (Fig. 2C) and a small increase in firing number and frequency after the addition of histidine (Fig. 2G). On the other hand, in a representative well of saline buffer, the

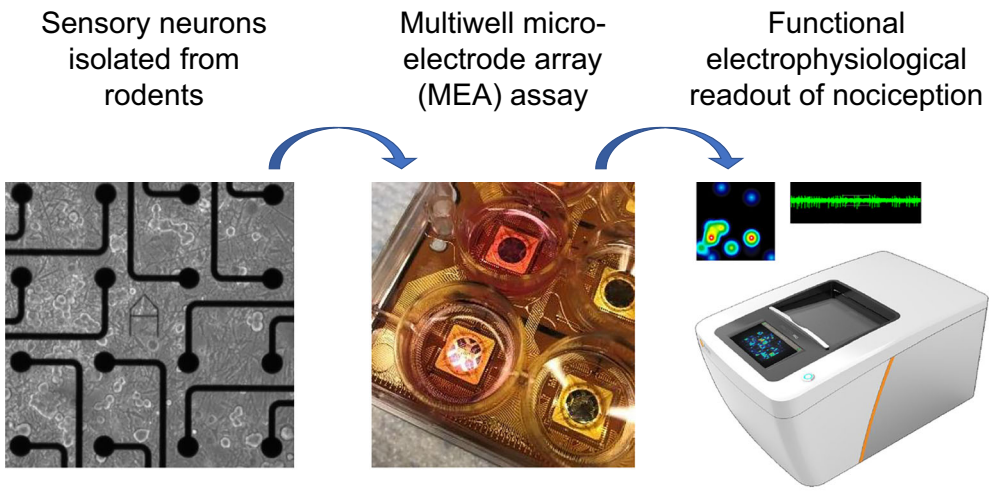

Fig. 1 *Diagram of MEA-based in vitro assay*. Dorsal root ganglia (DRG) sensory neurons were isolated from rats, then grown on the multi-electrode array plate. After culturing, we recorded the responses of the DRG sensory neuron after buffer addition as a functional electrophysiological readout of nociception.

addition of saline did not change the number of active neurons or overall firing frequency (Fig. 2D and H).

The representative temporal raster plot likewise shows an increase in firing events after buffer addition (Fig. 3). Each short black line indicates one action potential firing activity. In a representative raster plot for citrate salt, the baseline has 15 spontaneously firing neurons with one being more active with a higher firing frequency (Fig. 3A). After the addition of citrate-salt buffer, there is a slight delay, then the number of firing neurons increases, and the firing frequency also increases. The addition of citrate-mannitol buffer causes an immediate increase in firing neurons with a large increase in firing frequency as well (Fig. 3B). Notably, this major increase of neuronal firing seems to decrease after a while.

Histidine addition increases the number of firing neurons slightly, but does not seem to cause any identifiable increase in firing rate (Fig. 3C), which is similar to that observed after saline addition (Fig. 3D). Since some populations have spontaneous firing neurons as illustrated in the baseline of Figs. 2 and 3, we presented our results as an average fold change based on the individual unit's baseline before buffer addition, then aggregated by well. These representative figures (Figs. 2 and 3) qualitatively highlight that citrate buffers cause a larger increase in firing frequency after addition whereas histidine and saline only cause a slight increase. Noteworthy, citrate salt buffer caused an increased firing after a short delay, whereas citrate mannitol buffer caused an increase in firings more rapidly.

Quantitatively, we compared firing frequency, burst frequency, and burst duration before and after buffer addition as indicators of a nociceptive response. Firing of the neuronal culture before buffer addition was used as a baseline. Normal DRG media was used as a control. To summarize our data, we found that the citrate-salt and citrate-mannitol buffers had a significant increase in mean firing rate fold change (one-way ANOVA with Bonferroni corrections) when compared to the media control while histidine and saline did not significantly differ (Fig. 4A). The mean firing fold changes were 1.039 ± 0.019 (DRG culture media control), 1.736 ± 0.237 (citrate-salt, $p = 0.1487$ compared to DRG culture media control), 2.923 ± 0.366 (citrate-mannitol, $p < 0.001$), 1.382 ± 0.215 (histidine, $p = 0.9992$), and 1.067 ± 0.035 (saline, $p > 0.9999$).

Another measure of neuronal firing recorded by MEA is bursting events which include bursting frequency and duration. Citrate-mannitol was the only buffer with a significant

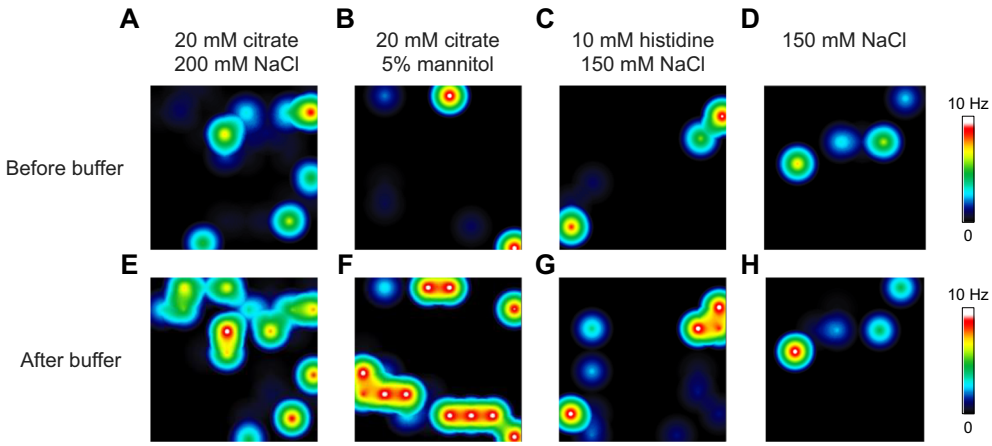

Fig. 2 *Heatmap of representative MEA recordings from DRG sensory neurons*. The firing frequency of each active electrode is color-coded: warm colors (red, orange, yellow) represent high firing frequency; cool colors (green, blue) represent low firing frequency. Each circle represents an active electrode of neuronal firing within the 8 × 8 electrode array. The top row (**A-D**) is representative images before buffer addition. The bottom row (**E-H**) is representative images after buffer addition.

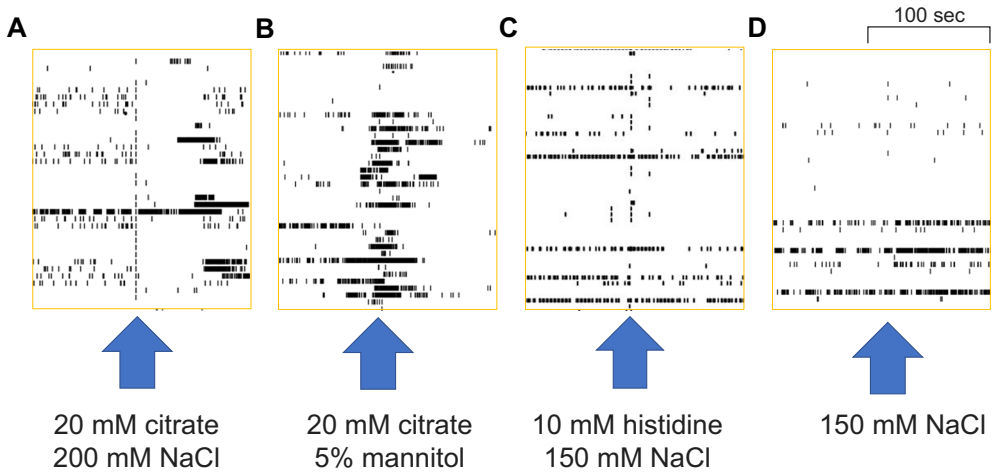

Fig. 3 *Temporal raster plot of representative MEA recording from DRG sensory neurons*. Each horizontal plot represents an active unit. Thick black horizontal bars represent action potential spikes. Blue bars represent burst firing (defined as ≥5 spikes over 100 ms). Arrows point at the time buffer was added to the well which led to some recording artifacts (vertically aligned plots at the arrow). (**A**) Citrate salt increased firing after a delay. (**B**) Citrate mannitol immediately caused the increased firing. (**C**) Histidine and (**D**) saline addition did not trigger much of a change from baseline.

increase in both burst frequency and burst duration (Fig. 4B and C). The burst frequency fold change was 1.001 ± 0.0004 (DRG culture media), 1.016 ± 0.018 (citrate salt, p > 0.9999), 1.055 ± 0.018 (citrate mannitol, $p = 0.0122$), 1.014 ± 0.012 (histidine, p > 0.9999), and 1.004 ± 0.002 (saline, p > 0.9999). The burst duration fold change was 1.038 ± 0.013 (DRG culture media), 1.509 ± 0.328 (citrate salt, $p = 0.3306$), 1.778 ± 0.062 (citrate mannitol, $p = 0.0359$), 1.220 ± 0.113 (histidine, p > 0.9999), and 1.246 ± 0.124 (saline, p > 0.9999). In ranking the buffers by the nociceptive response from most to least impactful based on the fold change over three parameters (mean firing rate, burst frequency, and burst duration), citrate mannitol causes the most increased firing, followed by citrate salt, then histidine, and lastly saline. Together, these data demonstrate that buffer systems with citrate, but not histidine or saline, could trigger higher DRG firing suggestive of a nociceptive response.

Reducing the Concentration of Citrate in the Buffers Lowers Firing in DRG Sensory Neurons

Low concentrations of citrate (10 mM) are currently used in some formulations including human growth hormone (25). We tested lower concentrations of citrate as further validation of the sensitivity of the system, which also helps to compare our findings with other results that measured injection site pain (26). The "low-citrate-salt" buffer consisted of 10 mM citrate and 150 mM NaCl. The "low-citrate-mannitol" buffer consisted of 10 mM citrate and 5% mannitol.

The mean firing rate, burst frequency, and burst duration were non-significantly lower in the 10 mM low-citrate-salt buffer as compared to the original 20 mM citrate-salt buffer and were closer to baseline (Fig. 5, blue bars). Citrate-salt buffer had a mean firing rate fold change of 1.736 ± 0.237, and that of the low-citrate-salt buffer was 1.116 ± 0.086 ($p = 0.4244$). Citrate-

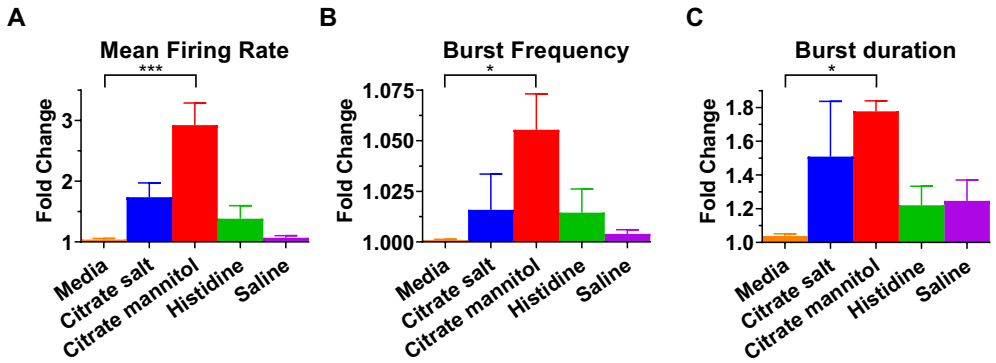

Fig. 4 *DRG sensory neuron firing change before and after addition of buffer*. (**A**) Mean firing rate increases after buffer addition, but not media or saline controls. Fold change was calculated by normalizing the data based on the baseline of the culture before buffer addition. (**B**) Burst frequency increases after buffer addition, but not media or saline controls. Burst firing is defined as ≥5 spikes over 100 ms. (**C**) Burst duration increases after buffer addition. Bars represent mean values with error bars in SEM. $p < 0.05$ (*) and $p < 0.001$ (***). Media $n = 6$, citrate-salt $n = 5$, citrate-mannitol $n = 6$, histidine $n = 7$, and saline $n = 5$ (wells).

salt buffer also had a burst frequency fold change of 1.016 ± 0.018, which was higher than that of the low-citrate-salt buffer of 1.005 ± 0.003 ($p > 0.9999$). The burst duration showed a change of 1.509 ± 0.328 for the citrate-salt buffer to 1.176 ± 0.077 for the low-citrate-salt buffer ($p > 0.9999$).

Notably, the low-citrate-mannitol buffer had a significantly lower mean firing rate, lower burst frequency, and lower burst duration as compared to the original citrate-mannitol buffer (Fig. 5, red bars). In particular, the citrate-mannitol buffer had a mean firing rate fold change of 2.923 ± 0.366, and that of the low-citrate-mannitol buffer was remarkably reduced to 1.109 ± 0.051 ($p < 0.001$). Citrate-mannitol buffer had a burst frequency fold change of 1.055 ± 0.018. For the low-citrate-mannitol buffer, the burst frequency was lower at 1.001 ± 0.0006 ($p = 0.0265$). Additionally, citrate-mannitol buffer had a larger burst duration fold change of 1.778 ± 0.062, and the low-citrate-mannitol buffer was 1.193 ± 0.034 ($p = 0.5437$). These results indicate that decreasing the concentration of citrate from 20 mM to 10 mM leads to a lower mean firing rate, burst frequency, and burst duration of DRG neurons firing patterns, suggesting a lesser nociceptive response of DRG neurons.

DISCUSSION

Nociception is the physiological process underlying the sensation of pain (27). *In vitro* DRG sensory neuron firing and bursting patterns (nociceptive responses) have been correlated with neuropathic pain (13,16,28). Furthermore, rat DRG neurons in an MEA platform have been used to evaluate the effects of ion channel modulators on neuron excitability (29). Using DRG sensory neuron based "nociception in a dish" model, here we evaluated four commonly used buffer systems and found that citrate-based buffers led to the highest increase in neuronal firing of DRG sensory neurons, suggesting that citrate-based buffers may have the highest nociceptive potential. Histidine was found to have a slight increase in firing compared to saline. Our findings are largely consistent with human clinical studies (25,26,30,31).

Mannitol is suggested to have a potential analgesic effect on peripheral nerve pain (32–35). We observed a greater nociceptive response from the 20 mM citrate with 5% mannitol formulation than the 20 mM citrate with 200 mM NaCl. However, a recent clinical trial suggests that citrate with mannitol is less painful than citrate with NaCl buffer (26). This difference between our *in vitro* model and clinical studies could be due to the nature of our reductionist system that only contains a primary culture of rodent DRG neurons. This discrepancy could also be due to the difference between nociception and the perceived pain. Interestingly, from our raster plot data (Fig. 3), we observed a delayed increase in firing of DRG neurons when exposed to citrate salt, whereas citrate mannitol triggers an immediate increase in neuronal firing. To be consistent in our analysis, we did not attempt to manually adjust the analysis time to account for this delay window. Thus, the relatively lower firing of neurons exposed to citrate-salt buffer compared to citrate-mannitol buffer could be partially due to the delay of firing increase, which also suggests that the pattern of neuronal firing might be another parameter to explore as a surrogate of nociception modeling. Nevertheless, more studies are needed to further clarify mannitol's role in molecular nociception and perceived pain with different experimental models and clinical studies.

One limitation of our study is that while we change the buffer components, the osmolarity changes as well. Although osmolarity has been suggested to affect the firing of neurons and nociception (36,37), it is difficult to decouple osmolarity from the effects of the buffer components to distinctly define the role of osmolarity by itself. Another contributor to nociception and injection site pain is inflammation. In rodents, subcutaneous injection of formalin is suggested to induce long-lasting peripheral inflammation (38). In humans, inflammation in non-neuronal cells can lead to pain amplification and hypersensitivity from the secondary release of

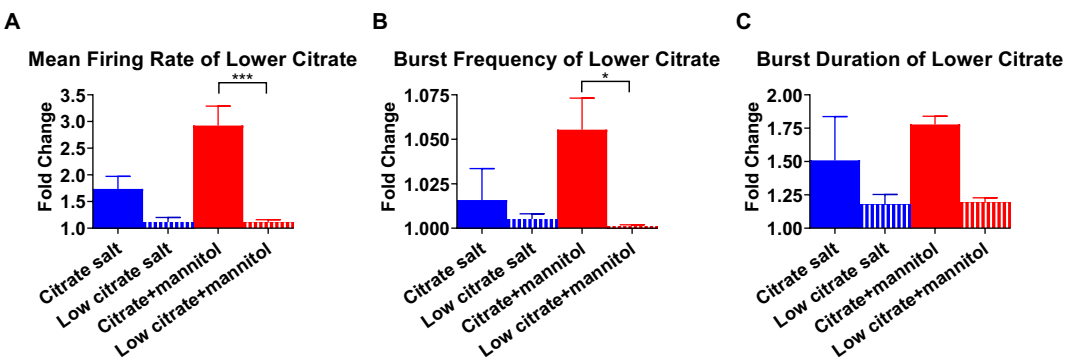

Fig. 5 *DRG sensory neuron firing change from buffers with lower concentrations of citrate*. (**A**) Mean firing rate was lower for low-citrate buffers compared to citrate buffers. Fold change was calculated to normalize the data based on the baseline of the culture before buffer addition. (**B**) Burst frequency was lower for low-citrate buffers. Burst firing is defined as ≥5 spikes over 100 ms. (**C**) Burst duration was lower for low-citrate buffers. Bars represent mean values with error bars in SEM. Solid bars are regular citrate buffers and striped bars are low citrate buffers. Blue bars are regular and low citrate-salt buffers. Red bars are regular and low citrate-mannitol buffers. $p < 0.05$ (*) and $p < 0.001$ (***). Citrate-salt $n = 5$, low-citrate-salt $n = 6$, citrate-mannitol $n = 6$, and low-citrate-mannitol $n = 6$ (wells).

inflammatory factors (39), which was not the focus of this study. However, since the primary rodent DRG culture was not neuronally pure and contained glial cells, there could be some proinflammatory factors released. Other factors including pH, temperature, volume, and speed of injection (40), can also potentially play a role in affecting the neuronal firing. However, the scope of our study focused on ranking major clinical buffer systems in terms of firing patterns.

MEA recording provides a high throughput approach to assess neuronal firing patterns and thus can serve as one of the first steps on the pipeline of characterizing the nociception effects of buffer systems to identify the optimal formulation. This method can be used to test temperature, pH, and other chemical factors of pharmaceutical buffer formulations. Initial screening could then be paired with whole-cell patch-clamp recording to provide detailed pharmacodynamics as the next step before proceeding to animal models or clinical trials. After screening in rodent DRG neuron-based assay, the results can be further validated in human DRG culture (41) or *in vivo* peripheral recordings in rodents.

CONCLUSION

We have studied four buffer systems commonly used in injectable medicine formulations with *in vitro* MEA assay. In conclusion, we show that clinically relevant buffer systems with high concentrations of citrate cause an increase in DRG firing higher than that caused by histidine or saline buffer systems. Our study demonstrates a robust *in vitro* method of measuring DRG sensory neurons firing/activity using MEA to serve as a surrogate of a nociceptive response to buffer systems. This assay could also help to understand the mechanism underlying pharmaceutical buffer related nociception.

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AUTHOR CONTRIBUTIONS

M.E., Z.Q., and J.Z. performed research and analyzed data; J.M. provided critical reagents; K.B., M.L., R.S., and J.M. contributed to the experimental design and data interpretation; M.E., M.L., and Y.Y. designed the project. Y.Y. supervised the project; M.E. wrote the paper with inputs from all authors.

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