The Effects of Sodium Chloride, Potassium Chloride, and Monosodium Glutamate on EaHy.926 Fish Epithelial Cells

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ABSTRACT

The increase in the levels of major ions, like Na+ and K+ has become a significant environmental issue on a global scale. The phenomenon, known as freshwater salinization, is considered one of the most significant aggravation that impact the biodiversity of freshwater ecosystems.This research aimed to investigate the effects of different concentrations of NaCl, KCl, and monosodium glutamate (MSG) on cells' ability to recover after a scratch assay. The percentage of cell recovery was analyzed as a direct measurement of cells' adaptation to stress. The results showed that at 2.5M, MSG was the least toxic treatment, with no growth but delayed lysis and detachment of cells. At 0.25M, MSG was also the least toxic treatment, with recovery similar to the control. At 0.025M, KCl was the treatment that most closely resembled the control. At 0.0025M, MSG was again the treatment that most closely resembled the control. NaCl did not show significant growth or recovery in any of the concentrations tested. Overall, the results suggest that MSG is less toxic to cells than NaCl and KCl at concentrations tested in this experiment, however, further studies may be required to fully evaluate the effects of MSG on different cell types and experimental conditions.

INTRODUCTION

Secondary salinization and the addition of metal ions to waterways is an urgent ecological issue in the world currently. It is defined as an increase in concentration of major ions, such as Na+ and K+, and is recognized as one of the top stressors on freshwater biodiversity (Cañedo-Argüelles et al., 2013; De Castro-Català et al., 2015). Causes of freshwater salinization include mining for other resources, agriculture, and road-deicing salts (Cañedo-Argüelles et al., 2013). Detrimental and permanent effects have been seen at organismal and population levels; however, more recently, studies have been done to assess the cellular and molecular levels (Cañedo-Argüelles et al., 2013). Human impacts interact with anthropogenic inputs of salts in a synergistic way that can lead to extreme changes in water quality – salinity, pH, nutrients and metals – creating the so-called 'freshwater salinization syndrome' (Kaushal et al., 2021). Assessing complex mixtures of major ions simply by analyzing physicochemical properties in the waterbody is not enough to determine toxicity levels at the cellular level (Cañedo-Argüelles et al., 2013). Unless the chemical nature of salt contamination is known and relatively stable they are inadequate metrics for regulation (Cañedo-Argüelles et al., 2013). Salt concentrations above 0.025M have been found to be toxic to marine life (Cañedo-Argüelles et al., 2013). Another metric that is worth looking at is the effect of commonly consumed salts from the human diet, such as monosodium glutamate (MSG). MSG is a flavour enhancer, typically used in Asian cuisine (Xiong et al. 2009). Its toxic nature may, in part, be mediated by its effect on neuron connections in the brain; however, this is understudied and is unknown at the cellular level (Xiong et al. 2009).This study aims to assess how cells recover after being exposed to varying concentrations of NaCl, KCl, and MSG in a scratch assay.

It is hypothesized that the percentage of cells recovered after the scratch will decrease with increasing concentrations of salt or acid. Assessing wound recovery will give us direct

measurements of how the cells adapt to stress factors and provide some insight as to how these organisms are affected in the ecosystems.

MATERIALS AND METHODS

Chemicals and equipment used:

• Ea.hy926 endothelial cells (provided by Science department, Thompson Rivers University)

• Cell culture medium (Leibovitz's L-15 + 10% fetal bovine serum (FBS))

• Fetal bovine serum (FBS)

• Cell culture flasks (T25-25cm^2, T25-75cm^2 flasks with a flat bottom and screw-top closure)

• Sodium chloride (NaCl) (provided by Science department, Thompson Rivers University)

• Monosodium glutamate (MSG) (provided by Science department, Thompson Rivers University)

• Potassium chloride (KCl) (provided by Science department, Thompson Rivers University)

• Vial of cells (5 x 105-1 x 106 cells from liquid nitrogen storage in a screw-top vial)

• Trypsin EDTA solution (0.5% trypsin, 5.3 mM Na4EDTA) for sub-culturing

• Bench top centrifuge capable of holding 15ml tubes

• Antibiotic/antimycotic solution (10,000U of Penicillin G, 10,000 µg streptomycin sulfateper ml)

• Invitrogen EVOS M5000 - digital inverted benchtop microscope

• Phosphate Buffered Saline (PBS)

Preparation of Endothelial Cell Culture Medium:

A 100 ml of Leibovitz's L-15 medium supplemented with either 10% fetal bovine serum (FBS) was prepared, as shown in Table 1. Antibiotic/antimycotic solution was added to the medium to prevent bacterial and fungal contamination.

The resulting 100 ml of complete medium was then filtered using a sterile filter to ensure sterility of all constituents. To achieve this, a syringe filter was used twice with a 50 ml syringe. The filtered medium was then transferred to 250 mL autoclaved glassware.

Table 1: Preparation of 100ml of Leibovitz's L-15 with FBS.

Constituents	**10% FBS**
Leibovitz's L-15	89 ml
Antibiotic/antimycotic	0.6 ml
Fetal growth serum	10 ml

The complete medium was stored at 4°C until needed. Prior to use, the medium was warmed to 37°C in the water bath. The medium was used for both feeding and sub-culturing endothelial cells.

Feeding cells:

Cells were maintained in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (FBS) and antibiotic/antimycotic solution. The cells were fed every 48-72 hours by removing half of the old medium and replacing it with fresh medium.

Incubation:

Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2.

Sub-culturing cells:

Ea.hy926 endothelial cells were sub-cultured using standard techniques. When cells approached confluence, the medium was removed, and the cells were washed with phosphate buffered saline (PBS) to remove any residual serum. Next, 2 mL of trypsin EDTA solution was added to the flask to detach the cells, which were then transferred to a 15 mL tube, centrifuged, and resuspended in complete medium. A cell count was performed using a hemocytometer and microscope, and the cells were then seeded into new T25 flasks at a ratio of 1:2 or 1:3 with a seeding density ranging from 200,000 to 300,000 cells in each flask. The flasks were placed in an incubator at 37°C.

Scratch Assay:

The scratch assay was performed to evaluate the effect of NaCl, MSG, and KCl on cell migration. Experiment was performed using two 24 well plates. The cells were grown to confluence and then seeded in each well at a suitable density. A linear cross scratch was made using a sterile 200 µl pipette tip. The cells were washed twice with PBS to remove the debris and treated with different concentrations of the test compounds. The plates were incubated for

24-48 hours, and images were taken at regular intervals (12-24-48 hours) to monitor cell migration.

RESULTS

Figure 1 shows the mean percent recovery of each scratch at 2.5M compared to the control treatment. The first thing to notice is that the control treatment experienced rapid growth, and the scratch was fully recovered after 24hrs. NaCl and KCl had moderate growth after 12hrs, but after 12 hours, the cells lysed and detached from the well. The 2.5M MSG did not experience any growth, but the cells did not lyse and become detached until after 24hrs. The data suggests that at 2.5M, MSG appears to be the least toxic treatment.

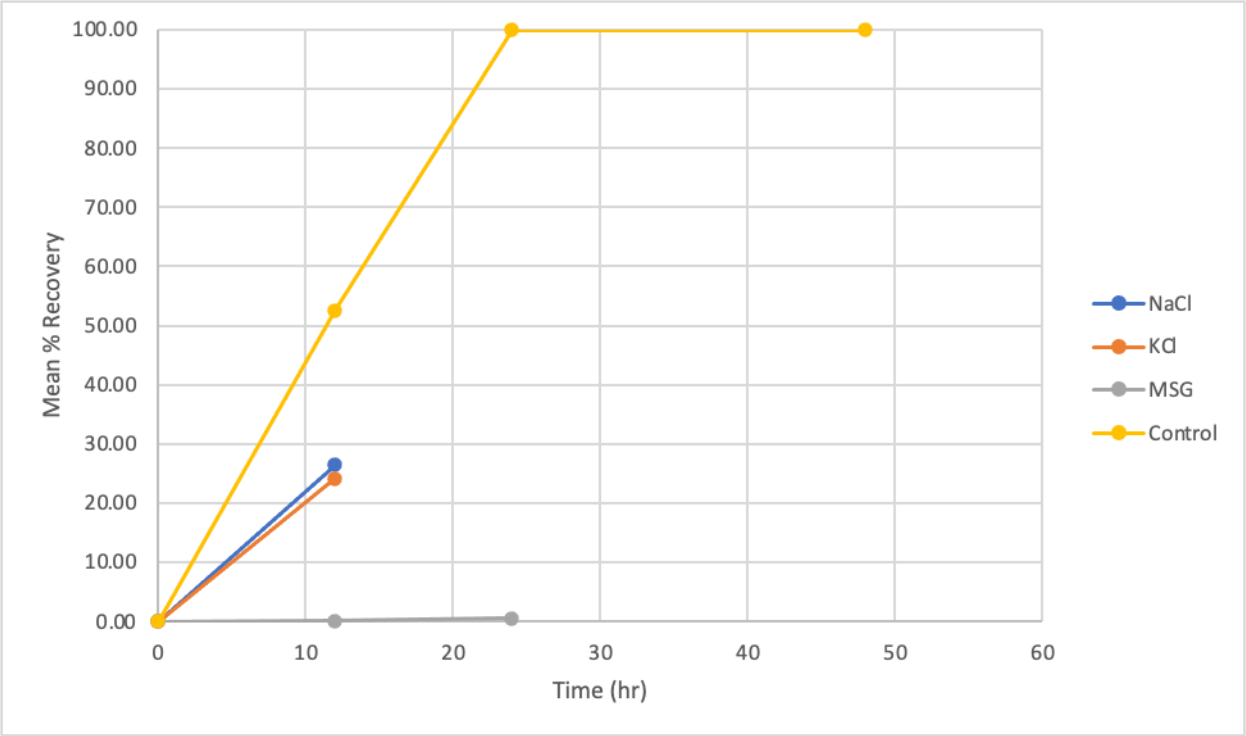

Figure 1 shows the mean percent scratch recovery over time for 2.5M solutions compared to the control treatment (n=3).

Figure 2 shows the mean percent recovery of each scratch at 0.25M compared to the control treatment. The control treatment here is the same as for the previous treatment and will be the same in subsequent treatments. In this treatment, the NaCl and KCl wells didn't experience any significant growth, and sometime after 24hrs, the NaCl cells lysed and detached from the well. The cells in the MSG well, on the other hand, experienced similar growth to the control after 12hrs, with recovery becoming less rapid after that, finishing the experiment with an average recovery of 89.4%. The data suggests that at this 0.25M, MSG is the least toxic to the cells.

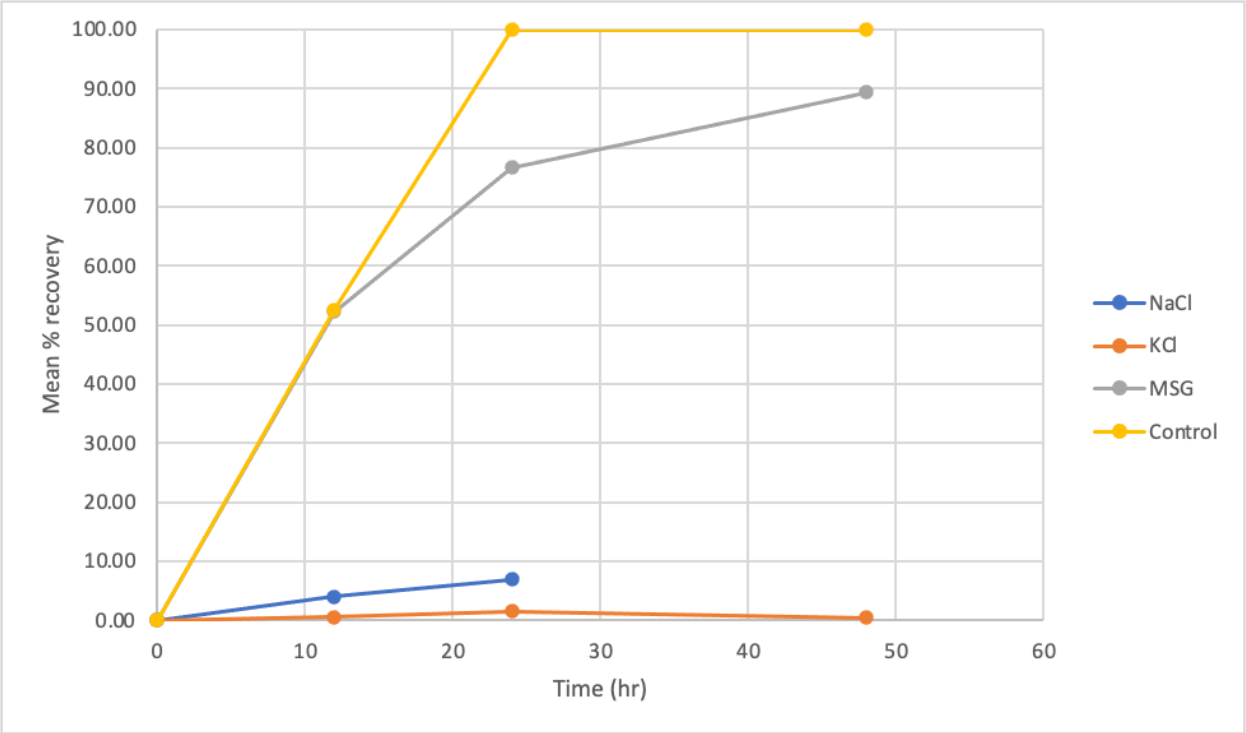

Figure 2 shows the mean percent scratch recovery over time for 0.25M solutions compared to the control treatment (n=3).

Figure 3 shows the mean percent recovery of each scratch at 0.025M compared to the control treatment. In this treatment, KCl had a similar growth rate to the control after 12hrs, and MSG and NaCl both had moderate growth rates. However, after 24hrs, the MSG wells had fully recovered, the NaCl wells had an average recovery of 96.1%, and the KCl wells had an average recovery of 96.7%. After 48hrs all three treatments were fully recovered. The data suggests that at 0.025M, KCl is the treatment that most closely resembles the control.

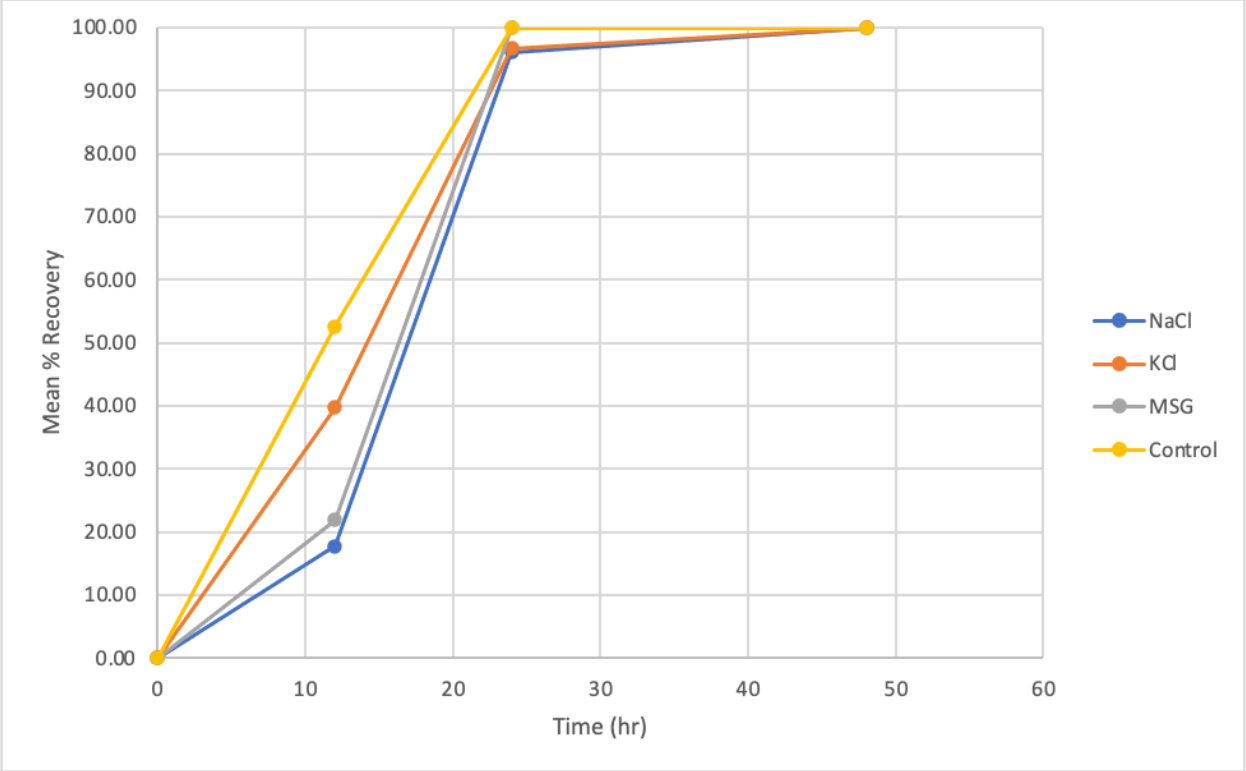

Figure 3 shows the mean percent scratch recovery over time for 0.025M solutions compared to the control treatment (n=3).

Figure 4 shows the mean percent recovery of each scratch at 0.0025M compared to the control treatment. The MSG and KCl wells recovered at a similar rate as the control after 12hrs, and the NaCl well recovered at a relatively moderate rate up to that point in time. After 24hrs, MSG the MSG scratches had fully recovered, the NaCl wells had an average recovery of 90.4%, and the KCl wells had an average recovery of 83.7%. After 48hrs, all three treatments had fully recovered. The data suggests that at this 0.0025M, the treatment that most closely resembles the control is MSG.

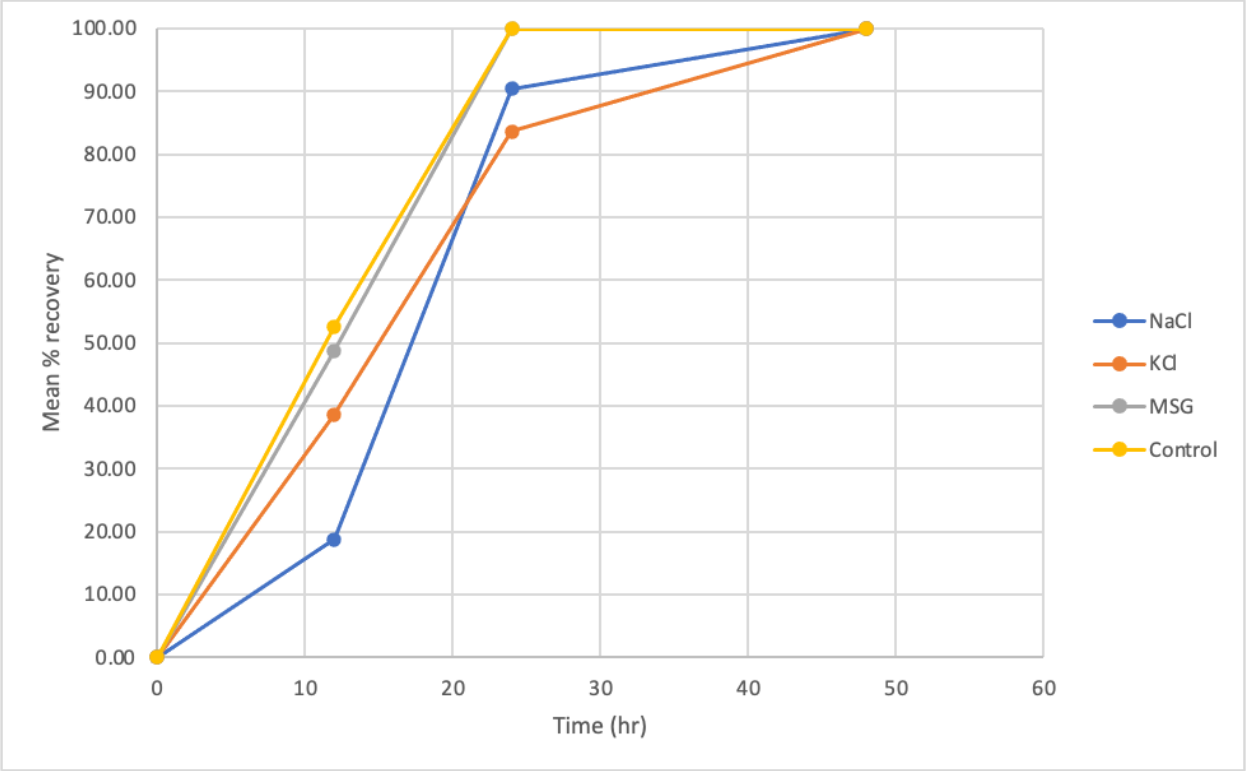

Figure 4 shows the mean percent scratch recovery over time for 0.0025M solutions compared to the control treatment (n=3).

Figure 5 shows the growth of one of the control wells after 24 hrs compared to time zero. The scratch in this case was 296.2mm, and had grown back fully after 24hrs. A few small yeast colonies can be seen growing on the right hand side of the scratch, but the colonies did not grow considerably. Although the large majority of the wells were free of contamination, this was typical of the wells that did have contamination. Occasionally, a small yeast colony was present, but the yeast colonies never grew to a considerable size.

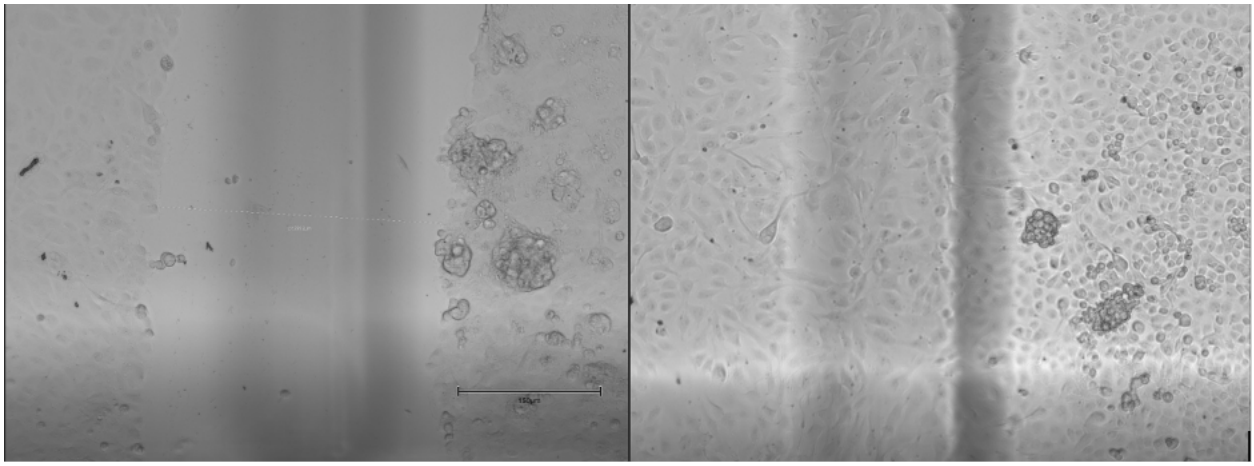

Figure 5 shows the control treatment at time zero (left) compared to time 24hrs (right).

DISCUSSION

In this study, the effects of NaCl, KCl, and MSG on EaHy.926 fish epithelial cells were assessed using a scratch wound assay. It was found that in all four treatment groups (2.5M, 0.25M, 0.025M, and 0.0025M) the cells had the highest recovery rate after the MSG treatment when compared to the control; cells were fully recovered after 48 hrs. It is also evident that both the NaCl and the KCl treatments had a greater negative impact on the cells as they had lower percent recovery values when compared to both the control and MSG treatments, as suggested by Cañedo-Argüelles et al. and De Castro-Català et al.

This is supported by the study conducted by Cañedo-Argüelles et al. that found that salt concentrations of greater than 0.25 M cause great stress to marine life (2013). Our study suggests that this happens at a cellular level and at a much lower concentration. Results also partially support our hypothesis as the percentage of cells recovered after the scratch decreased with increasing concentrations of salt or acid. However, after 48 hrs this could not be assessed as the cells had fully recovered in most trials.

During our experiment we were limited by time and resources to fully assess the effects of sodium chloride, potassium chloride, and monosodium glutamate on fish endothelial cells. The scratch test was a convenient way to test cell migration and get a general idea of how the three compounds affected the growth of the cells, however, in the future, an MTT assay would give a more precise analysis of the effects these compounds have on cell growth.

Since these are all very well characterized and common compounds, the future research is limited, especially for sodium chloride and potassium chloride. Since sodium chloride is such a critical component of food, and it is chronically over consumed, leading to health problems such as strokes and cardiovascular diseases, studies are being conducted to find safe alternatives to sodium chloride (Rybicka and Nunes 2022). One of these compounds being tested as a safe alternative is potassium chloride, whose average intake is below the daily recommended value. The upper limit for potassium intake has not yet been established, and is currently being investigated (van Buren et al. 2016).

Finally, monosodium glutamate is an area of more active research. It is generally accepted as being safe, although hypersensitivity allergies often referred to as “Chinese restaurant syndrome”, and links to increased pain sensitivity, and atopic dermatitis have been reported. However, research has suggested that there is no real correlation between these ailments and MSG, and that the health problems associated with MSG have been based on excessive dosage. Future research directions for MSG include its impact on nociception and

fetal neurodevelopment, and following chronic exposure to dietary doses (Zanfirescu et al. 2019).

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APPENDIX

Data for Mean % Recovery…

2.5M

		Mean % recovery		
time (hr)	NaCl	KCl	MSG	Control
0	0	0	0	0
12	26.4103735	24.1791481	0.13136133	52.5669115
24	x	x	0.48011895	100
48	x	x	x	100

0.25M

		Mean % recovery		
time (hr)	NaCl	KCl	MSG	Control
0	0	0	0	0
12	4.03010726	0.51695585	52.236684	52.5669115
24	6.85080609	1.52827201	76.6791476	100
48	x	0.4670493	89.4027545	100

0.025M

		Mean % recovery		
time (hr)	NaCl	KCl	MSG	Control
0	0	0	0	0
12	17.7043778	39.7534356	21.8709476	52.5669115
24	96.1016207	96.7337471	100	100
48	100	100	100	100

0.0025M

		Mean % recovery		
time (hr)	NaCl	KCl	MSG	Control
0	0	0	0	0
12	18.7411584	38.6028542	48.7048481	52.5669115
24	90.4269679	83.653148	100	100
48	100	100	100	100