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The effect of common addictive substances on microtubule polymerization dynamics

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Abstract

Substance addiction can lead to major social, emotional, economic, and health consequences. Though they tend to possess less of an addictive potential than highly regulated drugs, it is important to understand the effect that easily accessible addictive substances have on the body. Our objective was to investigate the effect caffeine, alcohol (ethanol), and sugar (glucose) have on our cells, specifically directly on microtubules. Microtubules are a component of the cytoskeleton, and their proper function is essential for maintaining cell shape and movement, such as cell division. Microtubules are created by the polymerization of α-tubulin and β-tubulin into long filaments. We hypothesized that caffeine, ethanol, and glucose would alter the level of microtubule polymerization in a cell-free system. Using a fluorescence-based tubulin polymerization assay, we examined the effect of various concentrations of these substances on microtubule polymerization curves by comparing to a negative water control. For the tested substance concentrations and water control, we measured the slope and lag time for the linear increasing section of the normalized polymerization curve and compared them using one-way ANOVA followed by Tukey's multiple pairwise comparison test. We found that microtubule polymerization was inhibited for all tested concentrations of caffeine, as we observed significantly reduced polymerization rate and increased lag time. The 0.1% and 0.5% ethanol concentrations also inhibited polymerization, evidenced by a decreased polymerization rate, though not reaching significance, and a significantly increased lag time. In contrast, the 5% ethanol concentration promoted polymerization with significantly increased polymerization rate and reduced lag time. Glucose did not have a meaningful effect on microtubule polymerization. Given the essential role microtubules play in the cell, investigating the effects these accessible addictive substances have on microtubule dynamics is necessary to better understand the risks these substances pose.

Keywords

Addiction, Caffeine, Ethanol, Glucose, Microtubules, Tubulin, Polymerization, Cytoskeleton, Cell-Free System, Fluorescence

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Introduction

Substance addiction can lead to major consequences for an individual and their community. There are major social, emotional, economic, and health burdens associated with addiction (1). Due to these negative consequences, highly addictive substances tend to be tightly regulated and difficult to access. In this study, we chose to focus on easily accessible addictive substances, such as caffeine, alcohol, and sugar, even though they have less of an addictive potential than the more regulated substances. Caffeine is a natural stimulant, whose consumption is widespread due to its ability to increase alertness and concentration (2). Caffeine is even considered to be the most widely consumed psychoactive stimulant in the world (3). Excessive caffeine consumption can increase risk of dehydration, headaches, insomnia, and anxiety (2).

Alcohol, composed mainly of ethanol, is a psychoactive substance commonly consumed in many forms across the world (4). The consumption of alcohol can develop into an addiction that leads to excessive intake. In fact, as of 2021, 29.5 million people ages twelve and over had Alcohol Use Disorder, characterized by uncontrolled drinking, in the past year in the United States (5). The negative effects of long-term alcohol consumption are well known. Excessive alcohol consumption can increase risk of high blood pressure, stroke, cardiovascular disease, chronic respiratory disease, and liver disease (6-10). There is also an increased risk of certain types of cancer from excessive alcohol intake. Chronic alcohol use is a risk factor for liver cancer and, even at low levels, leads to an increased risk of breast cancer (11, 12). There is also a heightened risk of oral, laryngeal, pharyngeal, esophageal, and colorectal cancers (9, 13, 14). In addition, the risk of learning and memory problems, including dementia and cognitive performance decline, increases with alcohol overconsumption (15).

Though often not considered an addictive substance, studies have shown that sugar has an addictive potential under some circumstances because its consumption leads to the release of dopamine and the stimulation of opioid systems in the brain (16). Overconsumption of glucose can be a risk factor for high blood pressure, cardiovascular disease, obesity, type II diabetes, and non-alcoholic fatty liver disease (17-19). The widespread consumption of caffeine, ethanol, and glucose makes it important to begin to understand the effects these substances may have on the body, whether those be negative effects to be avoided or characteristics that can be harnessed to improve health. A lot of research has been done on the effects these commonly consumed addictive substances have on full organ systems (5, 20, 21). Less research has been done on the direct effect these substances have on specific mechanisms within the cell. Simplifying the system to examine the effect of these substances on just one critical cellular mechanism can help isolate direct effects from indirect effects. This is necessary if we hope to understand exactly how caffeine, ethanol, and glucose result in the changes that we observe on the cellular, tissue, and full body levels.

One critical cellular mechanism that has been explored to a limited degree in the context of these addictive substances is microtubule growth dynamics. Microtubules are cellular structures that are an important component of the cytoskeleton. Microtubules are composed of α-tubulin and β-tubulin heterodimers that polymerize into hollow microtubule filaments (22). It is currently accepted that microtubule

formation begins with a thermodynamically unfavorable nucleation phase where tubulin heterodimers initially bind longitudinally (head-to-head) to form short protofilaments (23, 24). The binding of guanosine triphosphate (GTP) to tubulin allows these protofilaments to transition from a bent to a straightened conformation, which allows protofilaments to form lateral (side-to-side) interactions (24, 25). The addition of these lateral interactions produces a rectangular sheet of tubulin dimers (25). Once the sheet reaches a critical size it is able to close into a tube and provide a platform for thermodynamically favorable microtubule elongation, ending the nucleation phase (22-24). During the elongation phase, both single tubulin heterodimers and tubulin oligomers bind to the microtubule nucleus that was formed, causing the microtubule to grow (23). Microtubules are dynamic, meaning they undergo constant rapid assembly and disassembly. Differences in the stability of microtubules with GTP-bound tubulin and guanosine diphosphate (GDP)-bound tubulin contribute to this dynamic nature. GTP-bound tubulin promotes the growth and stability of the microtubule (25). However, GTP hydrolyzes to GDP, and GDP-bound tubulin promotes microtubule disassembly (25). Thus, as long as GTP-tubulin is added to the microtubule faster than disassembly occurs due to GDP-tubulin, the microtubule increases in length (23). This microtubule growth continues until the microtubule enters the equilibrium state when the number of tubulin heterodimers released equals the number of heterodimers gained (22). Microtubules provide structural support and help create the shape of eukaryotic cells along with serving as pathways for cellular protein transport. Additionally, microtubules play an important role in controlling cell movement, which is critical for the process of cell division and also for the functioning of movement-reliant cells such as neurons. Due to the ubiquitous nature of caffeine, alcohol, and glucose consumption, it is important to determine whether these substances significantly affect microtubule polymerization by interacting directly with microtubule assembly dynamics in an extracellular system. By examining the effect of these addictive substances on microtubules in an extracellular system, we reduce the influence of confounding factors, such as interactions with associated proteins, other cellular components, tissues, and organs.

Within cells, many proteins interact with microtubules to regulate their growth and dynamics. Prior research in plant cells shows that caffeine has an inhibitory effect on cytokinesis (26, 27), a stage of cell division that relies heavily on microtubules. This indicates that caffeine potentially disrupts microtubule polymerization in the cell though it is unclear if this occurs directly or through interactions with microtubule-associated proteins (MAPs). It has been reported that prolonged ethanol abuse leads to the disruption of cellular migration, cell signaling, and neurogenesis, which are all highly microtubule-dependent processes (28). Glucose, at high concentrations, induces microtubule remodeling through rapid microtubule assembly and disassembly, but this process occurs through mediation by a MAP (29). Thus, the objective of this study was to investigate the effects of these addictive substances in a cell-free system to isolate the effects on tubulin dynamics from the effects on MAPs. We hypothesized that caffeine, ethanol, and glucose would alter the level of microtubule polymerization in a cell-free system.

Methods

Fluorescence-based tubulin polymerization assay

A fluorescence-based tubulin polymerization assay (Cytoskeleton, Denver, CO) using over 99% pure tubulin from porcine brain (2 mg/mL) was employed. In this assay, polymerization was followed by fluorescence as a fluorescent reporter was incorporated into the microtubules as they polymerized. 100 µg of tubulin was used per assay with a 50 µL volume of reaction. The various concentrations of each of the three agents, caffeine, ethanol, and glucose, were prepared, with caffeine and glucose dissolved in water and mixed, before being placed on ice (Table 1). 5 µL of each agent concentration was pipetted in replicates of three onto the bottom of a half area opaque black 96 well plate (Corning Costar, Corning, NY). Three replicates of 5 µL of water were used as a negative control. General tubulin buffer (80 mM PIPES pH 6.9, 2.0 mM $MgCl_2$, 0.5 mM EGTA, 10 µM fluorescent reporter), GTP stock (100 mM), tubulin glycerol buffer (80 mM PIPES, 2.0 mM $MgCl_2$, 0.5 mM EGTA, 60% glycerol pH 6.9), and tubulin stock (10 mg/ml) were thawed by hand and put on ice along with the 96 well plate. Four sets of reaction mixes were prepared on ice. Each set contained 292 µL of general tubulin buffer, 134.4 µL of tubulin glycerol buffer, 5.28 µL of GTP stock, and 102 µL of tubulin stock. The assay components were mixed, incorporating the tubulin last, and 45 µL were pipetted quickly onto the wall of each well, avoiding bubbles. Fluorescence was measured by the Synergy 2 fluorescence plate reader (BioTek Instruments, Winooski, VT) at 37 °C with excitation at 360 nm, emission at 460 nm, and readings every minute for 130 minutes.

Table 1. Agents and their concentrations evaluated in the experiment

Agent	Concentrations			
Caffeine	0.1 mM	0.25 mM	0.5 mM	1 mM
Ethanol	0.1%	0.5%	1%	5%
Glucose	5 mM	10 mM	20 mM	

Statistical Analysis

Statistical analysis was conducted using R software (30). Fluorescence data obtained from each of the three replicates of the tested agent concentrations and the water control were normalized by dividing by the corresponding initial fluorescence value. We chose to normalize by the initial fluorescence value because, although the concentration of tubulin was constant for the various agents tested, there may have been differences in the initial degree of polymerization for tubulin added to each sample. Studies show that larger tubulin oligomers polymerize faster than smaller tubulin oligomers since larger oligomers have more landing sites for tubulin to bind either longitudinally or laterally (25). Thus, differences in the initial degree of polymerization, indicated by initial fluorescence values, could affect polymerization dynamics, thus, justifying the approach to normalize to the initial fluorescence values. Graphs of the normalized and averaged fluorescence data were visually inspected. Next, to characterize polymerization dynamics, the characteristic parameters (maximum slope and lag time) of the linear increasing section of the polymerization curve were estimated for each replicate using the linear fit algorithm in the fl.workflow function in the "QurveE" package (31). Lag time was estimated as the intersection between the tangent to the linear increasing section of the curve and the horizontal line with $y = y_0$, where y_0 is the lowest fluorescence value

for the replicate. To obtain linear regression results that accurately represent only the section of the curve that was linearly increasing, we used the default R^2 threshold of 0.97 in fl.workflow and manually increased the size of the sliding window to 15 to cover a larger fraction of the polymerization curve. While 0.97 was the default R^2 threshold value applied in the analysis, the achieved R^2 fits were better, with the lowest R^2=0.983 and median R^2=0.999. Next, using one-way Analysis of Variance (ANOVA) we compared the estimated slope parameter between the water control and the agent concentrations for each agent separately. ANOVA was followed by the Tukey's multiple pairwise comparison test. The homogeneity of variances was tested by the Levene's test, and the normality of residuals was tested by the Shapiro-Wilk test. The comparison of the lag time parameters followed the same approach used in the analysis of the slope parameters. To visualize parameter differences between the agent concentrations and the water control, the slope and lag time values estimated for each replicate were first normalized to the water control by dividing by the mean slope or lag time for the water replicates. Then, for each agent concentration, these normalized slope or lag time values were used to generate boxplots, showing the median and range of parameter values among the replicates (plots generated using GraphPad Prism version 10.3 for Mac, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). Analyses of the curves' characteristic parameters were complemented with a permutation test of the difference between groups of curves, which enabled comparison of the entire curves. Namely, curves normalized and averaged at each time point across the three replicates were analyzed using the compareGrowthCurves function in the "StatMod" package (32), with 10,000 simulations and an offset of 0.5. In all analyses, statistical significance was tested at the 5% threshold.

Results

We examined the effect of various concentrations of caffeine, ethanol, and glucose on microtubule polymerization (Table 1), compared to the water control, using a fluorescence-based tubulin polymerization assay (see methods). The level of microtubule polymerization was determined by measuring fluorescence every minute for 130 minutes. In Figures 1, 2, and 3, time in minutes is shown on the x-axis, and normalized relative fluorescence is shown on the y-axis. The level of fluorescence, indicating the level of polymerization, at every time point, is shown for the different concentrations of each agent that we tested. A water negative control is indicated on each graph as the blue curve. On each graph, there is an initial decrease in fluorescence, indicating depolymerization. To our understanding, this initial decrease in polymerization has not been observed in studies evaluating microtubule polymerization dynamics. After a minimum fluorescence value is reached, there is a brief, seemingly parabolic, increase in fluorescence, which is characteristic of the nucleation stage of microtubule polymerization (24). During this stage, tubulin aggregates to form a seed from which the microtubule grows. Each graph then shows an approximately linearly increasing fluorescence, indicating polymerization, which is the elongation phase of the microtubules, followed by steady-state equilibrium at the end.

To characterize and compare polymerization dynamics, we extracted the slope and lag time for the linear increasing section of the normalized polymerization curve for each of the three replicates of each tested agent concentration and the water control. The slope

and lag time parameters were subjected to the ANOVA (with Tukey's post hoc test) to compare polymerization dynamics among the tested agent concentrations and the water control. A significant difference in the slope and/or lag time indicates differences in the formation of microtubules. Specifically, we proposed that polymerization could be considered inhibited if we observed a significant decrease in polymerization rate and a significant increase in lag time. Alternatively, polymerization could be considered to be promoted if we observed a significant increase in polymerization rate and a significant decrease in lag time.

Caffeine inhibits microtubule polymerization

All tested caffeine concentrations, 0.1 mM, 0.25 mM, 0.5 mM, and 1 mM caffeine, produced polymerization curves appearing less steep than the water control curve, with the elongation phase also appearing to start later, indicating a longer lag time (Figure 1). This visual observation was confirmed by statistical analysis. At all caffeine concentrations tested, microtubule polymerization was inhibited. We found a statistically significant difference in the slopes ($F(4,10)=14.9$, p-value=3.2×10^{-4}) and lag times ($F(4,10)=6.4$, p-value=8.1×10^{-3}) for the tested caffeine concentrations. A Tukey's post-hoc test revealed significant pairwise differences between each tested concentration and water for the slope parameter (all p-values≤0.01, Table 2) and the lag time parameter (all p-values≤0.03, Table 2). There was no evidence of violated assumptions of the homogeneity of variance (Levene's test for the slope parameter $F(4,10)=0.81$, p-value=0.55 and for the lag time parameter $F(4,10)=0.80$, p-value=0.55) and normality of residuals (Shapiro-Wilk test for the slope parameter p-value=0.13 and for the lag time parameter p-value=1.0).

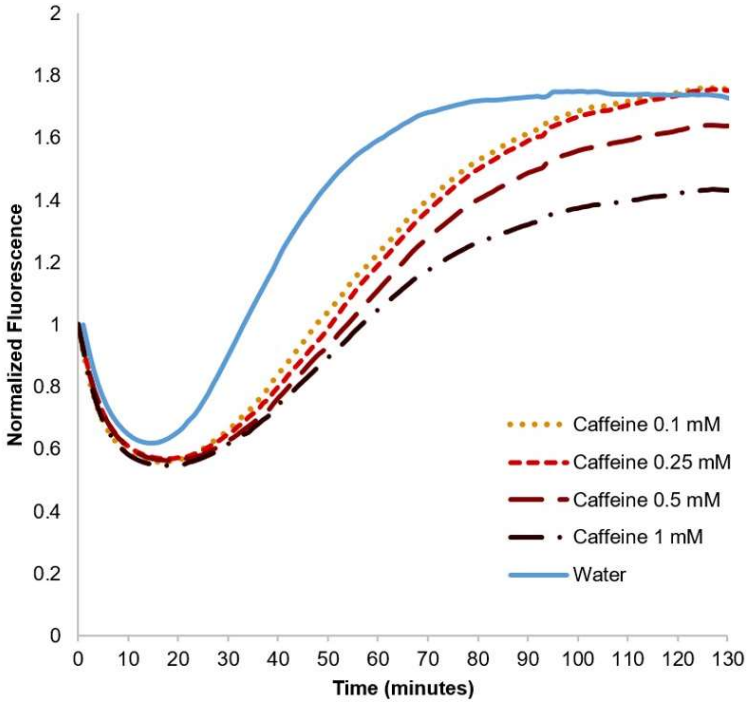

Figure 1. Polymerization curves for tubulin in the presence of various concentrations of caffeine or the negative water control (blue curve) measured through fluorescence over time. Shown relative fluorescence values were normalized by dividing the raw fluorescence values by the initial fluorescence value for each replicate and then averaging across replicates.

The 0.1 mM, 0.25 mM, 0.5 mM, and 1 mM caffeine concentrations all resulted in inhibited polymerization, i.e., a lower rate of polymerization per minute (diff = -9.5×10^{-3}, -1.1×10^{-2}, -1.3×10^{-2}, and -1.5×10^{-2}, respectively, Table 2, Figure 4) and longer lag time in minutes (diff=7.8, 9.1, 9.3, and 8.5, respectively, Table 2, Figure 5) compared to the water control. Complementing these results, in the permutation test (which compared the entirety of normalized polymerization curves averaged across replicates), the polymerization curves differed between each tested caffeine concentration and water; for concentrations 0.1 mM, 0.25 mM, 0.5 mM, and 1 mM caffeine compared to water, the p-values were 0.002, 5×10^{-5}, 5×10^{-5}, and 5×10^{-5}, respectively (Table 3). We found no statistically significant difference in the slope (p-values≥0.18, Table 2) and lag time (p-values≥0.96, Table 2) between any combination comparing the different caffeine concentrations to each other.

Low ethanol concentrations inhibit while high ethanol concentrations promote polymerization

We measured the level of microtubule polymerization in the presence of four concentrations of ethanol (0.1%, 0.5%, 1%, and 5%) over the course of 130 minutes. Surprisingly, compared to the water control, microtubule polymerization appeared promoted for 5% ethanol but inhibited for 0.1% and 0.5% ethanol (Figure 2). For 5% ethanol, the polymerization curve appeared steeper than the water curve; the process of microtubule polymerization appeared to be expedited as a whole, with equilibrium being reached at around 35 minutes for 5% ethanol but later, at approximately 100 minutes, for the water control. In contrast, the curves representing 0.1% and 0.5% ethanol concentrations appeared to begin the growth phase later and to have a slightly lower slope than the water control. This visual observation was confirmed by statistical analysis. Specifically, we found a statistically significant difference in the slope ($F(4,10)=101.1$, p-value=4.8×10^{-8}) and lag time ($F(4,10)=62.4$, p-value=4.9×10^{-7}) among the tested ethanol concentrations. A Tukey's post-hoc test revealed a significant pairwise difference between the 5% ethanol concentration and water in the slope parameter (p-value=4.1×10^{-7}, Table 2). For the comparisons of the 0.1% and 0.5% ethanol concentrations to water, the slope differences did not reach the level of statistical significance (p-values=0.10 and 0.16, respectively, Table 2). Tukey's post-hoc test also revealed significant pairwise differences between the 0.1%, 0.5%, and 5% ethanol concentrations and water in the lag time parameter (p-values≤0.02, Table 2). There was no evidence of violated assumptions of the homogeneity of variance (Levene's test for the slope parameter $F(4,10)=0.70$, p-value=0.61 and for the lag time parameter $F(4,10)=0.59$, p-value=0.68) and normality of residuals (Shapiro-Wilk test for the slope parameter p-value=0.47 and for the lag time parameter p-value=0.86).

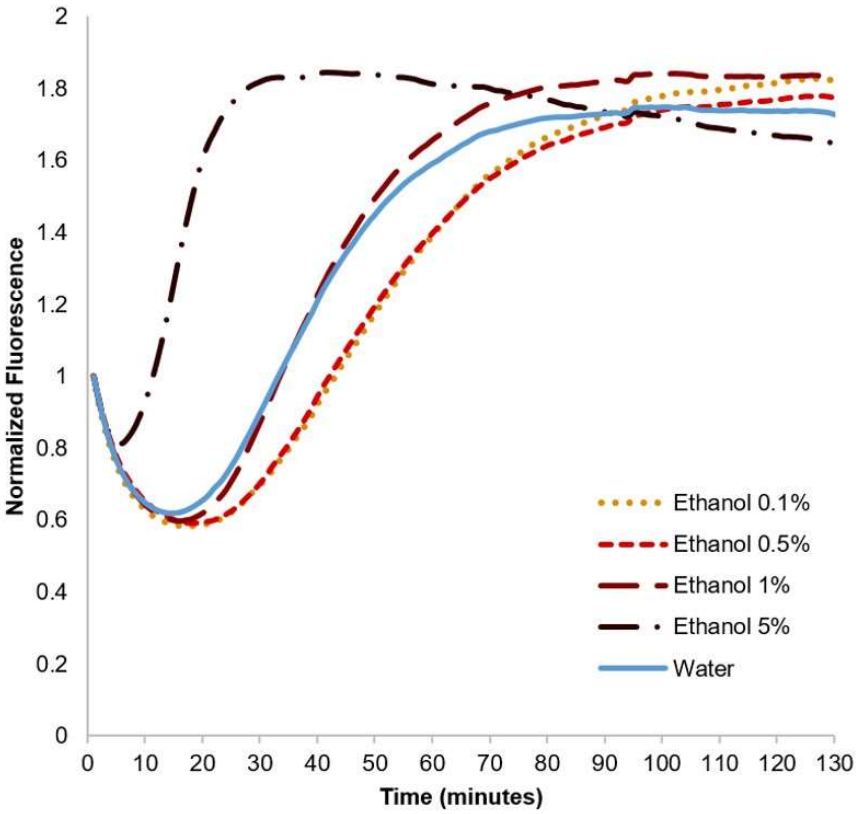

Figure 2. Polymerization curves for tubulin in the presence of various concentrations of ethanol or the negative water control (blue curve) measured through fluorescence over time. Shown relative fluorescence values were normalized by dividing the raw fluorescence values by the initial fluorescence value for each replicate and then averaging across replicates.

The 5% ethanol concentration resulted in a greater rate of microtubule polymerization per minute (diff=$2.9x10^{-2}$, Table 2, Figure 4) and shorter lag time in minutes (diff=-13.3, Table 2, Figure 5) compared to the water control. In contrast, the 0.1% and 0.5% ethanol concentrations demonstrated a lower rate of microtubule polymerization than the water control (diff=$-5.7x10^{-3}$ and $-5.1x10^{-3}$, respectively, Table 2, Figure 4) though the difference did not reach the level of statistical significance. Additionally, the 0.1% and 0.5% ethanol concentrations resulted in a significantly longer lag time in minutes (diff=5.5 and 5.7, respectively, Table 2, Figure 5) when compared to the water control. The results of the permutation test complemented the 5% ethanol findings as they showed that the polymerization curve for the 5% ethanol concentration was significantly different from the water control (p-value=$5x10^{-5}$, Table 3). We found a statistically significant difference in the slope (p-values≤0.01, Table 2) and lag time (p-values≤0.04, Table 2) between nearly all combinations comparing the different ethanol concentrations to each other. The only exceptions were the lack of significance for the ethanol 0.5% to 0.1% comparison (for both slope and lag time p-value=1.00, Table 2) and the borderline significance for the ethanol 1% to 0.1% lag time comparison (p-value=$5.1x10^{-2}$, Table 2).

Glucose does not significantly impact microtubule polymerization

Visual inspection of polymerization curves for tested glucose concentrations (5 mM, 10 mM, and 20 mM) indicated small differences in the polymerization dynamics (Figure 3). This result was confirmed by statistical analysis. We found no statistically significant difference in the slope parameter ($F(3,8)=2.1$, p-value=0.18). However, there was a borderline statistical difference in the lag time ($F(3,8)=3.8$, p-value=0.06), which a Tukey's post-hoc test revealed resulted from a borderline pairwise difference between the 10 mM glucose concentration and water (p-value=$4.5\text{x}10^{-2}$, Table 2). The permutation test supports these findings as it failed to detect a statistical difference between each tested glucose concentration and water (p-values≥0.48, Table 3). We found no statistically significant difference in slope (p-values≥0.39, Table 2) and lag time (p-values≥0.65, Table 2) between any combination comparing the different glucose concentrations themselves.

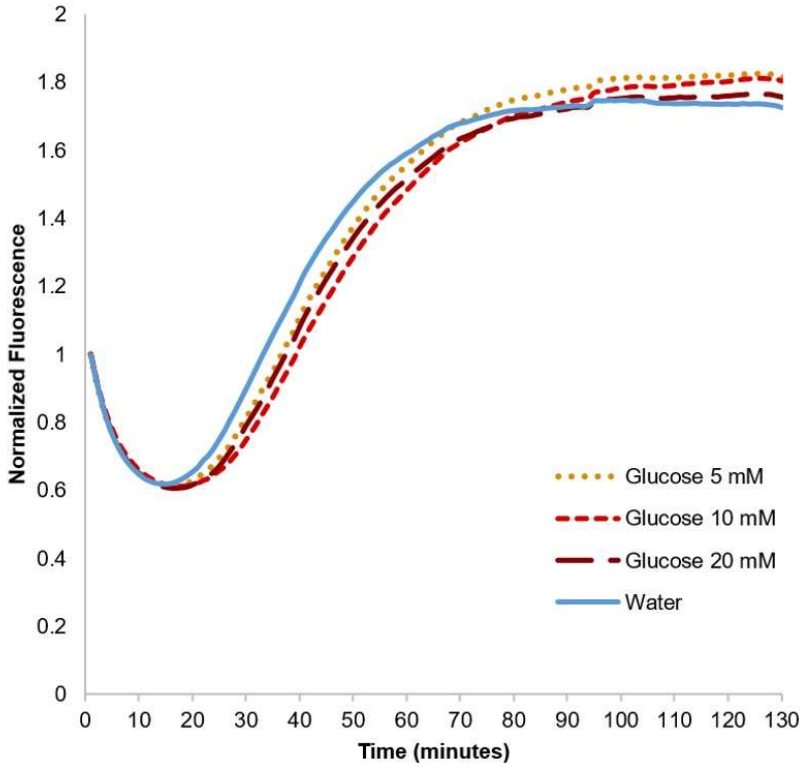

Figure 3. Polymerization curves for tubulin in the presence of various concentrations of glucose or the water negative control (blue curve) measured through fluorescence over time. Shown relative fluorescence values were normalized by dividing the raw fluorescence values by the initial fluorescence value for each replicate and then averaging across replicates.

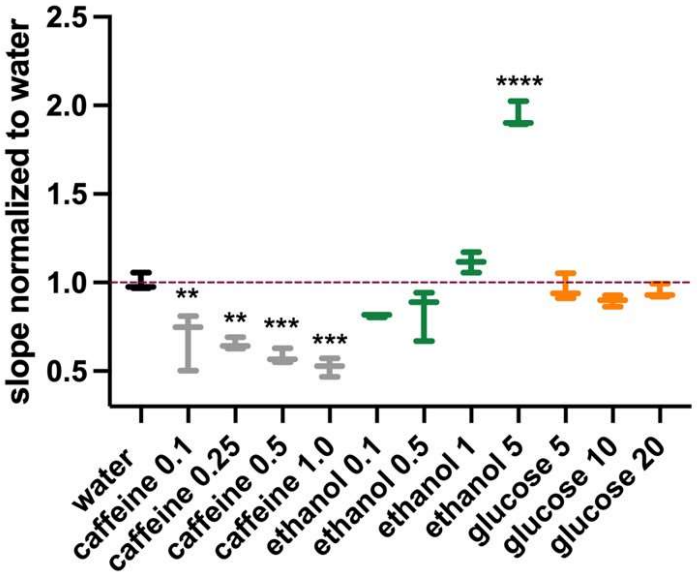

Figure 4. Slope describing the rate of change in fluorescence per minute (dF/dTime) for the linear increasing section of the normalized polymerization curves estimated for each replicate of the tested agent concentrations and the water control. Slope values were normalized to the water control by dividing the observed slope value by the average slope observed for the water control. For each tested agent concentration (mM) or water control, the boxplot shows the median (central horizontal line) and range (whiskers) fold difference to the average lag time for the water control. Asterisks denote statistical significance from the Tukey's test (≤0.0001=****; ≤0.001=***; ≤0.01=**; ≤0.05=*).

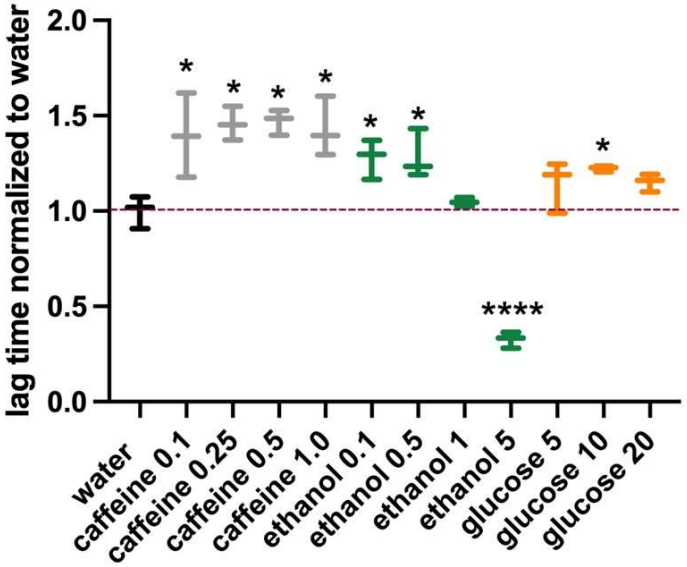

Figure 5 Lag times (in minutes) for the normalized polymerization curves estimated for each replicate of the tested agent concentrations and the water control. Lag time values were normalized to the water control by dividing the observed lag time value by the average lag time observed for the water control. For each tested agent concentration (mM) or water control, the boxplot shows the median (central horizontal line) and range (whiskers) fold difference to the average lag time for the water control. Asterisks denote statistical significance from the Tukey's test (≤0.0001=****; ≤0.001=***; ≤ 0.01=**; ≤0.05=*).

Table 2. ANOVA (and Tukey's post hoc test) comparison of the characteristic parameters of the polymerization curves. Significant p-values are shown in bold.

Comparison (agent 1 - agent 2)	Slope (dF/dTime)				Lag Time (Minutes)			
	Diff[1]	Lower[2]	Upper[3]	p adj[4]	Diff[1]	Lower[2]	Upper[3]	p adj[4]
Caffeine								
0.1 mM - Water	-9.5×10^{-3}	-1.6×10^{-2}	-2.7×10^{-3}	**0.01**	7.8	0.6	15.1	**0.03**
0.25 mM - Water	-1.1×10^{-2}	-1.7×10^{-2}	-3.8×10^{-3}	$\mathbf{3.2\times10^{-3}}$	9.1	1.8	16.3	**0.01**
0.5 mM - Water	-1.3×10^{-2}	-1.9×10^{-2}	-5.9×10^{-3}	$\mathbf{7.8\times10^{-4}}$	9.3	2.1	16.5	**0.01**
1 mM - Water	-1.5×10^{-2}	-2.1×10^{-2}	-7.7×10^{-3}	$\mathbf{2.6\times10^{-4}}$	8.5	1.3	15.7	**0.02**
0.25 mM - 0.1 mM	-1.0×10^{-3}	-7.8×10^{-3}	5.7×10^{-3}	0.99	1.2	-6.0	8.4	0.98
0.5 mM - 0.1 mM	-3.2×10^{-3}	-1.0×10^{-2}	3.6×10^{-3}	0.55	1.5	-5.8	8.7	0.96
1 mM - 0.1 mM	-5.0×10^{-3}	-1.2×10^{-2}	1.8×10^{-3}	0.18	0.7	-6.6	7.9	1.00
0.5 mM - 0.25 mM	-2.2×10^{-3}	-9.0×10^{-3}	4.6×10^{-3}	0.82	0.2	-7.0	7.5	1.00
1 mM - 0.25 mM	-4.0×10^{-3}	-1.1×10^{-2}	2.8×10^{-3}	0.36	-0.6	-7.8	6.7	1.00
1 mM - 0.5 mM	-1.8×10^{-3}	-8.6×10^{-3}	5.0×10^{-3}	0.90	-0.8	-8.0	6.4	1.00
Ethanol								
0.1% - Water	-5.7×10^{-3}	-1.2×10^{-2}	8.3×10^{-4}	0.10	5.5	0.9	10.1	**0.02**
0.5% - Water	-5.1×10^{-3}	-1.2×10^{-2}	1.5×10^{-3}	0.16	5.7	1.1	10.2	**0.02**
1% - Water	3.5×10^{-3}	-3.0×10^{-3}	1.0×10^{-2}	0.44	0.9	-3.6	5.5	0.96
5% - Water	2.9×10^{-2}	2.2×10^{-2}	3.5×10^{-2}	$\mathbf{4.1\times10^{-7}}$	-13.3	-17.9	-8.8	$\mathbf{1.8\times10^{-5}}$
0.5% - 0.1%	6.5×10^{-4}	-5.9×10^{-3}	7.2×10^{-3}	1.00	0.2	-4.4	4.7	1.00
1% - 0.1%	9.2×10^{-3}	2.7×10^{-3}	1.6×10^{-2}	**0.01**	-4.6	-9.1	0.0	0.05
5% - 0.1%	3.4×10^{-2}	2.8×10^{-2}	4.1×10^{-2}	$\mathbf{7.6\times10^{-8}}$	-18.8	-23.4	-14.2	$\mathbf{7.2\times10^{-7}}$
1%- 0.5%	8.5×10^{-3}	2.0×10^{-3}	1.5×10^{-2}	**0.01**	-4.7	-9.3	-0.2	**0.04**
5% - 0.5%	3.4×10^{-2}	2.7×10^{-2}	4.0×10^{-2}	$\mathbf{9.1\times10^{-8}}$	-19.0	-23.5	-14.4	$\mathbf{6.6\times10^{-7}}$
5% - 1%	2.5×10^{-2}	1.9×10^{-2}	3.2×10^{-2}	$\mathbf{1.4\times10^{-6}}$	-14.3	-18.8	-9.7	$\mathbf{9.6\times10^{-6}}$
Glucose								
5 mM - Water	-9.8×10^{-4}	-5.1×10^{-3}	3.1×10^{-3}	0.87	2.8	-1.5	7.1	0.24
10 mM - Water	-3.1×10^{-3}	-7.2×10^{-3}	9.7×10^{-4}	0.15	4.4	0.1	8.7	$\mathbf{4.5\times10^{-2}}$
20 mM - Water	-1.7×10^{-3}	-5.7×10^{-3}	2.4×10^{-3}	0.59	3.0	-1.3	7.3	0.20
10 mM - 5 mM	-2.1×10^{-3}	-6.2×10^{-3}	2.0×10^{-3}	0.39	1.6	-2.7	5.9	0.65
20 mM - 5 mM	-6.7×10^{-4}	-4.8×10^{-3}	3.4×10^{-3}	0.95	0.2	-4.1	4.5	1.00
20 mM - 10 mM	1.5×10^{-3}	-2.6×10^{-3}	5.6×10^{-3}	0.67	-1.4	-5.7	2.9	0.73

[1] difference between the slope or lag time parameters subtracting the second agent concentration (or water) parameter from the first concentration listed
[2] lower bound of slope or lag time parameter difference
[3] upper bound of slope or lag time parameter difference
[4] the adjusted p-value from Tukey's test

Table 3. Permutation test (using 10,000 simulations and an offset of 0.5) of the difference between normalized polymerization curves in groups 1 and 2 averaged across replicates. Significant p-values are shown in bold.

Group 1	Group 2	p-value
caffeine 0.1 mM	water	$\mathbf{2\times10^{-3}}$
caffeine 0.25 mM	water	$\mathbf{5\times10^{-5}}$
caffeine 0.5 mM	water	$\mathbf{5\times10^{-5}}$
caffeine 1 mM	water	$\mathbf{5\times10^{-5}}$
ethanol 0.1%	water	0.16
ethanol 0.5%	water	0.10
ethanol 1%	water	0.36
ethanol 5%	water	$\mathbf{5\times10^{-5}}$
glucose 5 mM	water	0.96
glucose 10 mM	water	0.48
glucose 20 mM	water	0.50

Discussion

We tested the effects of three common addictive substances on microtubule polymerization using a cell-free model. Our study showed that tubulin exposed to caffeine at concentrations of 0.1 mM, 0.25 mM, 0.5 mM, and 1 mM led to inhibited microtubule polymerization. The effect of ethanol on microtubule polymerization varied by concentration, with low concentrations of ethanol (0.1% and 0.5%) inhibiting microtubule polymerization while high concentrations (5%) unexpectedly promoted microtubule polymerization. Microtubule polymerization was unaffected by exposure to concentrations of glucose from 5 mM to 20 mM.

Caffeine inhibits microtubule polymerization

Studies have observed caffeine's inhibitory effect on microtubule polymerization. In one study by Hałas et al., human non-small cell lung cancer (CHO AA8) cells exposed to 10 mM and 20 mM caffeine concentrations showed microtubule depolymerization (33). This result supports our findings as it shows that caffeine inhibits microtubule polymerization both in cells and, as we observed, in a cell-free system. The caffeine concentrations of 10 mM and 20 mM used by Hałas et al. are substantially higher than the maximum 1 mM caffeine concentration tested in our study. To our knowledge, no studies have examined the effect of such lower caffeine concentrations on microtubule polymerization dynamics, particularly in a cell-free system. One study by Zhang et al. did find that a caffeine concentration of 1 mM caused the phragmoplast, a structure consisting of microtubules essential in plant cell division, to expand more slowly (34). The Zhang et al. study did not measure microtubule polymerization itself so its results cannot be entirely compared to our study. However, the reduced rate of phragmoplast expansion could potentially be explained by our finding that 1 mM caffeine reduces the rate of microtubule polymerization.

It is interesting to note that a study by Belmadani et al. exposed adult myocytes, which are muscle cells, to 10 mM caffeine and found no alterations to the microtubule network (35). In particular, Belmadani et al. tested whether Ca^{2+} increase induced by caffeine would disrupt microtubules (35). It has been shown that Ca^{2+} accumulation induces microtubule disassembly in neonatal myocytes (36). The suggested explanation for caffeine's lack of effect on the microtubule network in adult myocytes in the Belmadani et al. study was that myocytes develop resistance to the disruptive effect of Ca^{2+} accumulation as they mature, potentially due to changes in Ca^{2+}-regulated proteins (35). However, our study demonstrated that a 1 mM caffeine concentration was able to disrupt microtubule assembly directly in a cell-free system where Ca^{2+} could not have been responsible. Since adult myocyte microtubules were unaffected by caffeine, our study suggests that these cells must be resistant to caffeine's direct disruptive effect on microtubules in addition to the Ca^{2+} accumulation. Thus, our study indicates that there may be another mechanism at play by which microtubules in cells respond to caffeine that must be explored.

Our study was not designed to determine whether caffeine has a direct disruptive effect on both microtubule nucleation and elongation or if it only directly affects nucleation, which then results in changes to elongation. Future studies should investigate this question further by observing microtubule polymerization dynamics when tubulin is exposed to caffeine after the nucleation phase is complete. If caffeine introduced after nucleation has no effect on the resulting tubulin polymerization curve, this would show that caffeine only alters

tubulin polymerization by affecting the nucleation process. However, if caffeine added post-nucleation has an effect on the polymerization curve, that would suggest caffeine can directly influence both nucleation and elongation independently. To our knowledge, no such studies have been conducted.

In Figure 1, we observe a pattern suggesting that the later the nucleation ends, the slower the rate of subsequent polymerization. Studies investigating the mechanics of microtubule polymerization provide insight into potential explanations for why we may see a decrease in polymerization rate coupled with an increase in lag time. Caffeine could have a direct disruptive effect on microtubule polymerization by reducing affinities between tubulin heterodimers, which control both nucleation and elongation dynamics (25). In order for microtubule elongation to occur, it is necessary for the tubulin aggregate formed during nucleation to reach a critical size (24). If caffeine makes it more difficult for tubulin heterodimers to stay bound together, that could explain the increased length of nucleation, indicated by the lag time, and reduced rate of elongation (25). Additionally, the caffeine could reduce the stability or frequency of the formation of straight tubulin oligomers necessary for nucleation to transition to elongation. A reduction in the abundance of straight oligomers could increase the nucleation time, increasing the lag time (24). Less straight oligomers could also potentially explain the reduced rate of elongation. It has been observed that an increase in the proportion of straight tubulin oligomers, as produced by the Y222F tubulin mutation, increases not only the nucleation rate but also the polymerization rate (24). Thus, this finding suggests that if a compound conversely reduces the proportion of straight oligomers, it may decrease the nucleation rate, thereby increasing the lag time, and reducing the polymerization rate. Further testing of how microtubules in cells and in cell-free models respond to various concentrations of caffeine is required to confirm our findings and better understand the implications on cell function.

Ethanol's effect on microtubule dynamics appears concentration-dependent

Ethanol had a surprising impact on microtubule polymerization dynamics, appearing to inhibit microtubule polymerization at low concentrations but promoting polymerization at high concentrations. Lower concentrations of ethanol (0.1% and 0.5%) caused the lag time to significantly increase, and there was a decrease in the rate of polymerization, though it did not reach statistical significance (Table 2). Thus, polymerization was inhibited for these lower ethanol concentrations. However, high concentrations of ethanol (5%) significantly decreased the lag time and increased the rate of microtubule polymerization, indicating polymerization was promoted. Blood alcohol concentrations as high as 5% are likely not able to be reached in the human body, as studies have shown that a concentration of 0.355% can lead to fatal acute alcohol intoxication (37). Even though the 5% concentration is likely unattainable in living cells, we chose to include it in our study to see how microtubules might behave at an extreme ethanol dose.

Our findings concerning inhibited polymerization upon exposure to low ethanol concentrations are partially supported by another study utilizing a cell-free model (28). This study by Smith et al. used a turbidity assay to record the level of polymerization for tubulin exposed to various ethanol concentrations. By comparing the level of tubulin polymerization at

each time point, the study found that MAP-independent microtubule polymerization was inhibited upon exposure to ethanol concentrations of 25 mM to 75 mM (28). We observed inhibited polymerization for the 0.1% and 0.5% ethanol concentrations in our study, which correspond to about 17 mM and 86 mM, respectively. However, the Smith et al. study did not explicitly measure the lag time and polymerization rate from the tubulin polymerization curves generated in their study. Thus, we are not able to confirm our findings that lag time increased while polymerization rate decreased for the 0.1% and 0.5% ethanol concentrations. It is important to note that the Smith et al. study did observe complete abrogation of tubulin polymerization at the 75 mM concentration, while we saw inhibited but not completely prevented polymerization at our slightly higher 0.5% ethanol concentration. The reason for this difference is unclear and requires further research. Smith et al. proposed that the inhibited microtubule polymerization observed in their study could be the result of ethanol competing with tubulin binding sites, slowing microtubule nucleation, which might also explain the increased lag time observed in our study (28).

Studies exposing living cells to ethanol have also observed the inhibitive effect that relatively low concentrations of ethanol have on microtubule polymerization. One study by Kannarkat et al. exposed liver WIF-B cells to 50 mM ethanol and found impaired microtubule polymerization, evidenced by shorter microtubules and a lower percentage of polymerized tubulin (39). Kannarkat et al. suggested that this inhibited polymerization could be mediated by acetaldehyde, a product of ethanol metabolism (39). While WIF-B cells are able to form acetaldehyde from ethanol, this could not have occurred in our cell-free model. Thus, our findings suggest that ethanol has an underlying direct inhibitive effect on microtubule polymerization that does not involve acetaldehyde. To confirm whether this is the case, further testing is needed.

The Smith et al. study tested 100 mM and 500 mM ethanol concentrations as well (28), which were closer to our 1% and 5% ethanol concentrations, corresponding to about 170 mM and 860 mM, respectively. However, the study only tested these high concentrations with MAP-rich tubulin, while our study did not test tubulin bound with MAPs. Thus, their result, finding high ethanol concentrations to have no effect on MAP-rich tubulin polymerization (28), is not applicable to our study. To our knowledge, no study has examined microtubule polymerization with an ethanol concentration as high as 5%. Thus, we are not able to confirm our result that 5% ethanol promotes microtubule polymerization.

Similarly to caffeine, from our study, we are not able to determine if ethanol affects nucleation and elongation independently or if it only affects nucleation, which then subsequently alters elongation. Thus, we propose conducting a future study where ethanol is introduced after tubulin nucleation is complete. The results of this further investigation would shed light on the mechanism by which ethanol alters microtubule polymerization dynamics. Our observations that ethanol inhibits polymerization at low concentrations but promotes polymerization at high concentrations may indicate that the mechanism by which ethanol impacts tubulin polymerization is concentration-dependent. We suggest this concentration dependence be further tested in future studies.

Glucose has no significant effect on microtubule polymerization

No effect of glucose on microtubule polymerization was observed in this study, apart from a slightly higher, not meaningfully different, lag time for 10 mM glucose compared to the control. Thus, our hypothesis that glucose causes significant changes to microtubule polymerization is not supported by the results of our study. Other studies have found similar results, showing that glucose does not have an effect on microtubule polymerization at the concentrations we tested. In one study by Rivelli et al., erythrocyte (red blood cell) treatment with 5 mM glucose did not lead to an increase in tubulin polymerization, whereas 25 mM glucose treatment showed a 38% increase in microtubules in the polymerization state (40). Our result that 5 mM glucose does not significantly affect microtubule polymerization dynamics is supported by this study. The highest concentration of glucose that we tested, 20 mM, did not significantly alter microtubule polymerization either. Further testing of higher concentrations of glucose is required to determine if glucose can alter microtubule polymerization dynamics in a cell-free system. The Rivelli et al. results could be caused by glucose triggering microtubule growth through cellular pathways. Studies have found that glucose can induce microtubule growth from the Golgi apparatus (29). A possible explanation is that glucose regulates microtubule assembly and disassembly at the Golgi apparatus by cellular pathways that respond to high levels of glucose rather than the glucose itself impacting microtubule growth, which would complement our findings. Even though we did not find glucose to have a significant impact on microtubule dynamics in a cell-free system, further research is necessary to investigate the mechanism(s) by which glucose affects microtubules in the cell. This is important to better understand how cells may respond to high concentrations of glucose for prolonged periods, as would be the case for subjects with diseases such as diabetes.

Why might addiction be problematic?

Addiction could cause high enough doses of caffeine or alcohol, that impede microtubule polymerization, to be frequently present in the body. The resulting disrupted microtubule dynamics could be detrimental for cell, tissue, and bodily health. Microtubules play important roles in maintaining cell shape, protein trafficking, and mitosis (35). Disruptions in these key processes could be harmful in many cell types. However, microtubule disruptions are particularly detrimental in cells, such as neurons, whose function relies heavily on an exaggerated cell shape and intracellular transport made possible by microtubules. In neurons, microtubules support the development of long axons and the dendrites that form connections with other neurons to allow for the transfer of signals (41). In addition to maintaining neuron shape, microtubules act as pathways for transport proteins to carry cargo within the cell (41). Microtubules are so essential for transport within neurons that microtubule depolymerization in dopaminergic neurons impedes the transport of dopamine vesicles, causing their accumulation in the neurons and, ultimately, cell death (42). The death of these dopaminergic neurons has been linked to Parkinson's disease (42). Thus, it is evident how a disruption in microtubule dynamics could potentially lead to or promote the onset of disease.

Cardiomyocytes are another cell type whose function, contracting and relaxing to help pump the heart, significantly relies on the shaping and transport properties of microtubules (43). In

cardiomyocytes, microtubules help regulate contractile mechanics and electrical activity (43). In fact, altered microtubules contributing to cardiomyocyte contractile dysfunction have been linked with cardiovascular diseases (43). These are just two examples of how microtubule disruptions impact cell function and can promote the onset of disease. Excessive alcohol or caffeine consumption will not necessarily lead to the development of disease, but it is a potential risk that makes it important to further investigate the relationship between addictive substances and microtubule dynamics. A thorough understanding of how addictive substances affect microtubules is necessary to recognize the nature of the effect these substances have on the body and the risks that addiction may present.

Limitations

We had only three replicates for each concentration of each addictive substance tested. More replicates of each substance would increase confidence in our findings. Additionally, testing more concentrations of each substance would provide a more thorough understanding of how the dose of caffeine, ethanol, or glucose affects microtubule dynamics. We are particularly interested in further investigating the unexpected promoted microtubule polymerization for 5% ethanol. Testing more concentrations of ethanol with more replicates would aid in understanding ethanol's impact on microtubule dynamics. Since our study used a cell-free model, our findings do not take into account the effects that various MAPs and other cellular systems have on microtubule assembly. The cell-free model was useful in order to isolate the effects of each addictive substance directly on microtubule assembly itself. However, this also meant that our findings might not be representative of how each of the tested substances impacts microtubules in living cells. Thus, further research is necessary to thoroughly understand the effects caffeine, ethanol, and glucose have on microtubules in cells and how this may affect tissues, organs, and full organisms.

Conclusion

Our study suggests that caffeine, at concentrations of 0.1 mM, 0.25 mM, 0.5 mM, and 1 mM, inhibited microtubule polymerization in a cell-free system. A similar inhibitory effect was observed with relatively low concentrations of ethanol, 0.1% and 0.5%. Unexpectedly, tubulin exposed to a high concentration of ethanol, 5%, promoted microtubule polymerization. Glucose had no substantial effect on the rate of microtubule polymerization at concentrations ranging from 5 mM to 20 mM. These findings suggest that caffeine and ethanol have a direct effect on microtubule polymerization dynamics since our study was conducted using a cell-free model. Further research is needed to understand the exact mechanisms by which caffeine and ethanol affect microtubule polymerization. The disrupted microtubule polymerization caused by caffeine and ethanol has important implications for human health, as proper microtubule function is necessary for cell shape, division, transport, and motility. It follows that cell types highly reliant on specific shape, motility, or protein trafficking properties for their proper function can be particularly at risk from microtubule disruptions, potentially leading to the development of disease.

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