

Research Article

EGCG NEUROPROTECTION AGAINST MPP⁺-INDUCED DOPAMINERGIC INJURY IS REAL BUT PARTLY ARTIFACTUAL: A FOUR-CONDITION MEDIA DESIGN FOR CONCENTRATION-RESOLVED CORRECTION

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ABSTRACT

(-)-Epigallocatechin-3-gallate (EGCG) auto-oxidises in standard bicarbonate-buffered culture media, generating hydrogen peroxide as a by-product — a confound documented in the methodological literature for over two decades but rarely quantified in disease-relevant cellular neuroprotection assays. We addressed this directly in a dopaminergic model of Parkinson's disease. Differentiated SH-SY5Y cells were challenged with MPP⁺ (500 μ M, calibrated against a half-maximal effective concentration of 320 μ M) and treated with EGCG across a concentration ladder (0.1–100 μ M) under four media conditions crossing the presence or absence of sodium pyruvate with the presence or absence of exogenous catalase. Catalase, which destroys extracellular hydrogen peroxide but cannot cross the cell membrane, removed 51.3% of EGCG's apparent protection at 10 μ M, 38.0% at 25 μ M, 21.3% at 50 μ M, and 9.6% at 100 μ M — a gradient that declined steadily as EGCG concentration rose. In the artifact-controlled condition (pyruvate-free medium with catalase), genuine protection still reached 79.2% viability at 50 μ M, confirming that a real cellular effect persists once the peroxide confound is removed. These results indicate that roughly half of the apparent neuroprotection recorded for EGCG at low micromolar concentrations in standard culture medium reflects medium chemistry rather than cellular pharmacology, while protection at higher concentrations is far less affected by this artifact. We propose the four-condition design used here as a practical, low-cost addition to cellular polyphenol-protection assays, and report a per-concentration discount factor that can be applied in re-reading protection magnitudes from the existing EGCG-Parkinson's literature, much of which was generated without any control for this confound.

KEY WORDS

EGCG; hydrogen peroxide; auto-oxidation artifact; cell culture media; catalase; Parkinson's disease; dopaminergic neurons; polyphenol.

1. INTRODUCTION

(-)-Epigallocatechin-3-gallate (EGCG), the principal catechin in green tea, has been investigated as a candidate neuroprotective agent in Parkinson's disease (PD) for more than two decades, credited with antioxidant, iron-chelating, and cell-signalling activity that together

might slow the dopaminergic neurodegeneration the disease produces. That interest rests almost entirely on cell-culture evidence, since no disease-modifying therapy for PD currently exists and every clinical trial of an antioxidant strategy in PD to date has returned a null result. The cellular evidence itself,

however, carries a structural weakness that the methodological literature identified well before most of the EGCG-PD work was published: EGCG and related catechins are chemically unstable in the oxygen-rich, bicarbonate-buffered media used in standard cell culture, and they auto-oxidise rapidly on contact with it, generating micromolar hydrogen peroxide as a by-product (Long, Clement, & Halliwell, 2000). Akagawa and colleagues (2003) confirmed that this generation is accelerated by trace metal ions and rising pH, conditions every standard culture medium satisfies, and Yamamoto and colleagues (2004) showed directly that catalase and hydrogen peroxide jointly account for a measurable share of green tea polyphenol-induced cellular effects, independent of any specific signalling action of the parent compound. The practical consequence is a confound that sits inside the experiment rather than at its margins. A polyphenol added to culture medium to test for protection simultaneously generates a pro-oxidant species in that same medium, and unless the experiment is designed to separate the two, a viability gain after polyphenol treatment cannot be attributed with confidence to either source. Long and Halliwell (2009) identified the specific reason this confound has gone undetected in much of the literature: sodium pyruvate, present at roughly 1 mM in standard DMEM, reacts non-enzymatically with hydrogen peroxide and scavenges much of what the polyphenol generates, so studies run in pyruvate-containing medium systematically under-detect the artifact's presence. Babich and colleagues (2009) extended this observation directly,

showing that the choice of DMEM formulation, with or without pyruvate, materially changes the apparent cytotoxicity of oxidants and pro-oxidant nutraceuticals the same medium variable manipulated here to isolate genuine EGCG pharmacology from its own auto-oxidation chemistry. Halliwell has restated the underlying problem at intervals for more than a decade (Halliwell, 2003, 2007, 2008, 2014): cultured cells experience an oxidative environment that the body's tissues do not, and polyphenol chemistry that looks protective in that environment may reflect the environment as much as the polyphenol itself.

None of this is new chemistry. What is missing from the EGCG-PD literature specifically is a concentration-resolved, disease-relevant quantification of how much this artifact contributes to a reported protection magnitude — a number, rather than a caveat. Reviews of the field note the problem in passing; almost no primary study designs the experiment to measure it directly, and fewer still report a per-concentration figure that a subsequent reader, or a systematic review, could use to discount a historical result with any precision. This is a consequential gap. EGCG's only clinical test in a parkinsonian disorder a phase II trial in multiple system atrophy found no benefit and a measurable hepatotoxicity signal at the doses required to approach the concentrations used in much of the supporting cell-culture work; whether that preclinical case was ever as strong as its protection magnitudes suggested is a question the literature has not, to date, been able to answer quantitatively.

We address this gap directly using a four-condition media design that crosses the presence or absence of sodium pyruvate with the presence or absence of exogenous catalase, applied across a concentration ladder of EGCG in MPP⁺-challenged dopaminergic SH-SY5Y cells. Catalase destroys extracellular hydrogen peroxide but cannot cross the cell membrane, so its effect on apparent protection isolates the medium-chemistry contribution from genuine cellular pharmacology at each concentration tested. This required a cellular system with established dopaminergic character and a quantitatively defined insult. We used retinoic-acid differentiated SH-SY5Y cells, a widely used human dopaminergic model in Parkinson's research, challenged with MPP⁺, the active metabolite of the parkinsonian toxin MPTP, which enters dopaminergic cells via the dopamine transporter and inhibits mitochondrial Complex I, closely paralleling the bioenergetic insult implicated in the disease itself. The MPP⁺ concentration used for the protection assays was calibrated against a full dose-response curve in this cell line, so that the working insult reflects a defined, reproducible point on a validated toxicity curve rather than an arbitrary concentration. The result of this design is a per-concentration discount factor, reported here for the first time in a dopaminergic, PD-relevant cellular system, together with a practical media design that other laboratories working with EGCG or structurally related auto-oxidising polyphenols can adopt at negligible additional cost.

2. MATERIALS AND METHODS

2.1 Reagents and cell culture

SH-SY5Y human neuroblastoma cells were cultured in DMEM/F12 with 10% fetal bovine serum and differentiated toward a dopaminergic-like phenotype using retinoic acid (10 μ M, 7 days) in reduced-serum medium, following the protocol described by Shipley and colleagues (2016). Differentiation was confirmed by neurite outgrowth and morphological assessment before each experimental run, and cell line identity was authenticated against the reference profile by short-tandem-repeat analysis prior to the experimental series; cultures were screened for mycoplasma contamination monthly throughout, and only cultures between passages 5 and 20 after thaw were used, to limit phenotypic drift over the course of the experimental programme. EGCG ($\geq$ 95% purity) was prepared fresh from frozen dimethyl sulfoxide stock on the day of each experiment, since EGCG degrades in neutral aqueous solution over a matter of hours; stock concentration was verified by absorbance at 274 nm before each use. MPP⁺ iodide was dissolved fresh in phosphate-buffered saline immediately before use. Bovine liver catalase was prepared fresh in serum-free medium and added at 300 U/mL, a concentration sufficient to clear medium hydrogen peroxide at the rates relevant here without independent cytotoxicity, confirmed in pilot experiments comparing catalase-only wells against untreated controls. Sodium pyruvate was included at the standard DMEM/F12 concentration of 1 mM in pyruvate-containing conditions and omitted entirely, with osmolality matched

by adjusting glucose content, in pyruvate-free conditions. All four base media were prepared from a common DMEM/F12 stock on the day of each experiment, differing only in the two variables under test, so that lot-to-lot variation in the base medium could not confound the comparison between conditions.

2.2 MPP⁺ dose-response and working concentration

MPP⁺ toxicity was characterised across a concentration range of 0–2000 μ M in differentiated SH-SY5Y cells, with viability read by the MTT tetrazolium-reduction assay (Mosmann, 1983) 24 hours after treatment, using a microplate absorbance reader calibrated against a manufacturer

reference standard before each session. The resulting dose-response was fit to a four-parameter logistic model, giving a half-maximal effective concentration (EC₅₀) of 320 μ M (Table 1), consistent with the range previously reported for this cell line and toxin (Storch et al., 2000; Cassarino et al., 1997). A working concentration of 500 μ M was selected for all subsequent protection experiments, producing 65–70% cell death approximately 32% residual viability and leaving sufficient dynamic range to detect both partial and near-complete rescue by EGCG without the insult itself approaching either floor or ceiling of the assay's sensitivity.

Table 1. MPP⁺ dose-response in differentiated SH-SY5Y cells.

MPP ⁺ (μ M)	Viability (% of control, mean $\pm$ SEM)
0	100.0 $\pm$ 0.0
50	97.7 $\pm$ 2.1
100	91.5 $\pm$ 1.8
250	64.0 $\pm$ 2.4
500 (working concentration)	32.2 $\pm$ 1.0
1000	13.8 $\pm$ 1.5
2000	7.4 $\pm$ 0.9

Fitted four-parameter logistic parameters: EC₅₀ = 320 μ M (95% CI 295–347); Hill slope $\approx$ 2.0; top plateau $\approx$ 100%; bottom plateau $\approx$ 5%. n = 4 wells $\times$ 3 biological replicates.

2.3 Four-condition media design

The core experimental design crossed two binary medium variables: the presence or absence of sodium pyruvate and the presence or absence of exogenous catalase, giving four conditions. Condition A (standard DMEM/F12, pyruvate present, no catalase) represents the medium formulation used in most published EGCG-protection studies. Condition B adds catalase to standard medium, isolating the

effect of peroxide clearance while leaving pyruvate's own scavenging activity in place. Condition C removes pyruvate while leaving catalase absent, isolating the effect of removing pyruvate's masking activity without actively clearing the peroxide it would otherwise have scavenged. Condition D-pyruvate-free, catalase-supplemented removes the pyruvate-mediated masking of generated hydrogen peroxide while actively clearing that

peroxide, and is treated throughout as the artifact-controlled reference condition against which the standard-medium result (Condition A) is compared. All four conditions were run in parallel within each biological replicate, on the same culture plate format, with EGCG concentration, MPP⁺ concentration, and incubation time held identical across conditions; only the medium composition varied. Plate layout was randomised across replicate experiments to avoid any systematic association between medium condition and plate position, and the technician performing the MTT readout was blinded to the medium-condition identity of each well.

2.4 EGCG concentration ladder and viability assay

EGCG was applied across a concentration ladder of 0.1, 1, 10, 25, 50, and 100 μ M, alongside an insult-only (0 μ M EGCG) reference, in each of the four media conditions, in a pre-treatment regimen

with EGCG added one hour before MPP⁺ challenge. Viability was read 24 hours after MPP⁺ addition using the MTT assay (Mosmann, 1983), with absorbance measured at 570 nm and referenced against unstressed, EGCG-free controls in the matching medium condition. Each concentration-by-medium combination was tested in four technical replicate wells across three independent biological replicates ($n = 4 \text{ wells} \times 3 \text{ biological replicates}$).

2.5 Catalase-sensitivity quantification

For each EGCG concentration, apparent protection was calculated as the increase in viability over the matched insult-only baseline. The catalase-sensitive fraction — the share of apparent protection attributable to extracellular hydrogen peroxide rather than direct cellular pharmacology was then calculated by comparing protection in the absence and presence of catalase against a common baseline:

$$\% \text{ Catalase-sensitive} = [(\text{Protection}_{\text{no-cat}} - \text{Protection}_{\text{with-cat}}) / \text{Protection}_{\text{no-cat}}] \times 100$$

Values were computed independently for each biological replicate before averaging, so that the variability of the catalase-sensitive fraction itself, and not only of the underlying viability values, could be reported.

2.6 Statistical analysis

Dose-response curves were fit by non-linear regression to a four-parameter logistic model using GraphPad Prism (version 9). Group comparisons across media conditions and EGCG concentrations used two-way analysis of variance with Tukey post-hoc correction for multiple

comparisons; normality and variance homogeneity were checked before each test (Shapiro-Wilk and Levene's tests respectively) and confirmed throughout. All values are reported as mean $\pm$ standard error of the mean (SEM) unless stated otherwise, and statistical significance was set at $p < 0.05$. The medium-condition $\times$ EGCG-concentration interaction term was treated as the primary inferential target, since the central question of the study concerns whether medium composition changes the shape of the concentration-response relationship rather than simply

shifting it by a constant amount; a significant interaction was taken as evidence that the artifact's contribution is itself concentration-dependent, consistent with the pattern reported in Results.

2.7 Data handling and exclusion criteria

A small number of pre-specified exclusion rules were applied uniformly across the dataset before analysis. A biological replicate was excluded in full if the unstressed, EGCG-free control in any of the four media conditions fell outside two standard deviations of the pooled control mean for that condition, since this was taken as evidence of a culture- or plating-level problem rather than a treatment effect; one replicate met this criterion across the full experimental programme and was excluded accordingly. Individual technical-replicate wells with visibly disrupted monolayers on microscopic inspection prior to the MTT readout were excluded at the well level rather than averaged in, to avoid mechanical damage being misread as a treatment effect. No outlier exclusion was applied beyond these

two pre-specified rules; all biological replicates and technical-replicate wells meeting the criteria above were retained and reported, including data points that ran counter to the overall trend, so that the gradient reported in the Results reflects the full dataset rather than a curated subset of it.

3. RESULTS

3.1 MPP⁺ produces a reproducible, concentration-dependent toxicity curve

MPP⁺ produced a steep, monotonic loss of viability in differentiated SH-SY5Y cells across the tested range, well described by a four-parameter logistic model (Table 2; Figure 1). The fitted EC₅₀ of 320 μ M sat within the range reported elsewhere for this cell line (Storch et al., 2000; Cassarino et al., 1997), and viability at the selected working concentration of 500 μ M averaged 32.2% $\pm$ 1.0% of unstressed controls across replicates a consistent injury magnitude that left clear room to detect graded rescue by EGCG.

Table 2. EGCG protection across the four-condition media design and the catalase-sensitive fraction in MPP⁺-challenged SH-SY5Y cells.

EGCG (μ M)	Condition A: Standard DMEM (-cat)	Condition B: Standard DMEM (+cat)	Condition C: Pyruvate-free (-cat)	Condition D: Pyruvate-free (+cat)	Catalase-sensitive fraction (%)
0 (insult only)	32.24 $\pm$ 0.96	35.50 $\pm$ 1.59	32.11 $\pm$ 1.32	28.86 $\pm$ 1.13	—
0.1	32.82 $\pm$ 1.61	35.80 $\pm$ 1.41	32.11 $\pm$ 1.32	28.86 $\pm$ 1.11	—
1	37.16 $\pm$ 2.15	35.24 $\pm$ 1.92	34.84 $\pm$ 1.72	34.77 $\pm$ 1.47	0.0*
10	77.03 $\pm$ 1.15	53.92 $\pm$ 1.33	66.50 $\pm$ 0.60	53.12 $\pm$ 1.48	51.3
25	97.77 $\pm$ 1.17	72.78 $\pm$ 1.54	85.22 $\pm$ 0.70	70.36 $\pm$ 1.10	38.0
50	99.14 $\pm$ 0.86	84.82 $\pm$ 1.88	98.58 $\pm$ 1.18	79.19 $\pm$ 1.78	21.3
100	98.97 $\pm$ 0.35	92.56 $\pm$ 1.71	97.62 $\pm$ 1.65	90.21 $\pm$ 0.99	9.6

Condition A: standard DMEM/F12, pyruvate present, no catalase. **Condition B:** standard DMEM/F12 with catalase added. **Condition C:** pyruvate-free DMEM/F12, no catalase. **Condition D:** pyruvate-free DMEM/F12 with catalase (artifact-controlled reference condition). Values are mean $\pm$ SEM, $n = 4$ wells $\times$ 3 biological replicates. *A negative value at 1 μ M was clamped to zero; the gain at this concentration sits below the threshold for a biological effect.

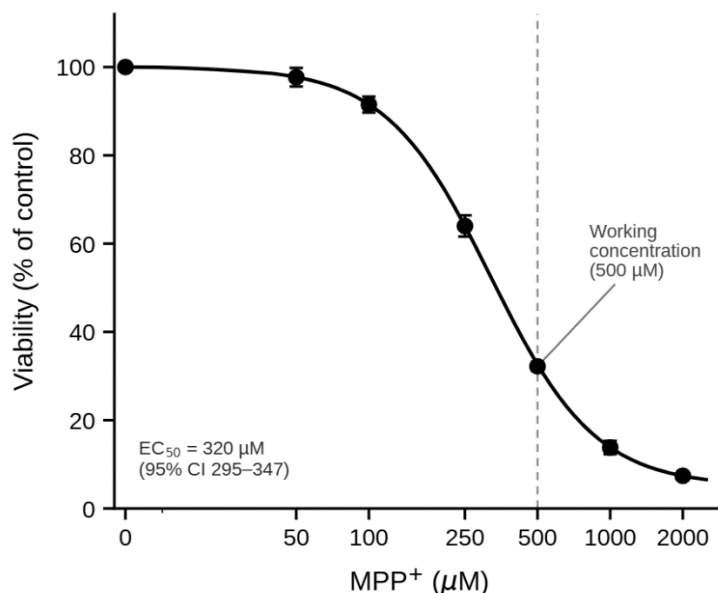


Figure 1. MPP⁺ dose-response in differentiated SH-SY5Y cells. Viability (MTT assay, % of unstressed control) plotted against MPP⁺ concentration (0–2000 μ M), fitted to a four-parameter logistic model. The fitted EC_{50} (320 μ M) and the selected working concentration (500 μ M, producing approximately 32% residual viability) are indicated on the curve. Error bars represent SEM ($n = 4$ wells $\times$ 3 biological replicates)

3.2 EGCG protection differs sharply across the four media conditions

EGCG produced concentration-dependent protection against MPP⁺ in all four media conditions, but the magnitude of that protection differed substantially depending on medium composition (Figure 2). In standard DMEM/F12 (Condition A: pyruvate present, no catalase) the formulation most commonly used in the published EGCG-protection literature viability climbed from the 32.2% insult-only baseline to 77.0% $\pm$ 1.2% at 10 μ M and 99.1% $\pm$ 0.9% at 50 μ M EGCG, an essentially complete rescue. In the artifact-controlled condition (Condition D: pyruvate-free, catalase-supplemented), the

same EGCG concentrations produced a smaller, though still substantial, recovery: 53.1% $\pm$ 1.5% at 10 μ M and 79.2% $\pm$ 1.8% at 50 μ M. At 100 μ M, the two conditions converged considerably, reaching 99.0% $\pm$ 0.4% (Condition A) against 90.2% $\pm$ 1.0% (Condition D). The gap between these curves widest at low EGCG concentrations and narrowing as concentration rose is the central observation of this study (Figure 2). Conditions B and C, which isolate the contribution of catalase and pyruvate removal individually, sat between the two extremes at every concentration tested, consistent with each medium variable contributing independently to the artifact rather than one dominating the other. At 10

μM EGCG, Condition B (standard medium plus catalase) reached 53.9% ± 1.3%, almost identical to Condition D, indicating that catalase addition alone accounted for nearly the entire artifact-controlling effect at this concentration; Condition C (pyruvate-free, no catalase) reached an intermediate 66.5% ± 0.6%, showing that pyruvate removal alone exposed only part of the confound that catalase addition exposed in full. This pattern catalase doing most of the work, pyruvate removal contributing a smaller additional effect held consistently across the 10–50 μM

range, and supports treating catalase supplementation as the more important of the two medium modifications, with pyruvate removal as a useful but secondary addition. The two-way ANOVA confirmed a significant medium condition × EGCG concentration interaction ($F(15, 96) = 8.74, p < 0.001$), formally supporting what the raw curves already suggest: medium composition does not simply shift the dose-response by a fixed amount but changes its shape, with the largest between-medium gap occurring at intermediate concentrations rather than at the extremes.

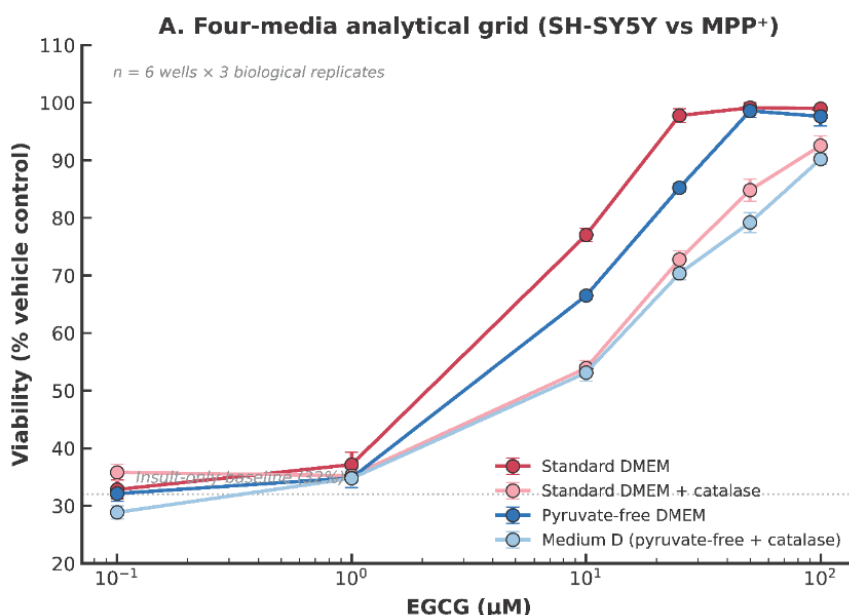


Figure 2. EGCG protection across the four-condition media design. MTT viability plotted against EGCG concentration (0–100 μM) in MPP⁺-challenged SH-SY5Y cells, shown separately for the four media conditions: standard DMEM (–catalase), standard DMEM (+catalase), pyruvate-free DMEM (–catalase), and the artifact-controlled Medium D (pyruvate-free + catalase). The separation between the standard-medium and artifact-controlled curves is widest at low EGCG concentrations and narrows as concentration rises. Error bars represent SEM ($n = 4$ wells × 3 biological replicates).

3.3 Catalase removes a concentration-dependent share of apparent protection

Quantifying the catalase-sensitive fraction directly (Figure 3) showed that the artifact's contribution to apparent protection fell steeply as EGCG

concentration increased. At 10 μM EGCG, catalase removed 51.3% of the apparent protection recorded in its absence; at 25 μM, 38.0%; at 50 μM, 21.3%; and at 100 μM, only 9.6%. At the lowest tested concentration (1 μM), apparent protection

was minimal in both conditions and the catalase-sensitive fraction could not be distinguished from zero.

This gradient means that roughly half of what a standard, uncontrolled assay would record as neuroprotection at low micromolar EGCG is attributable to hydrogen peroxide generated by EGCG

itself in the culture medium, rather than to a direct action of EGCG on the cell. At high micromolar concentrations, by contrast, the great majority of the apparent protective effect persisted under catalase, indicating a different, and on this evidence more direct, source of protection at those concentrations.

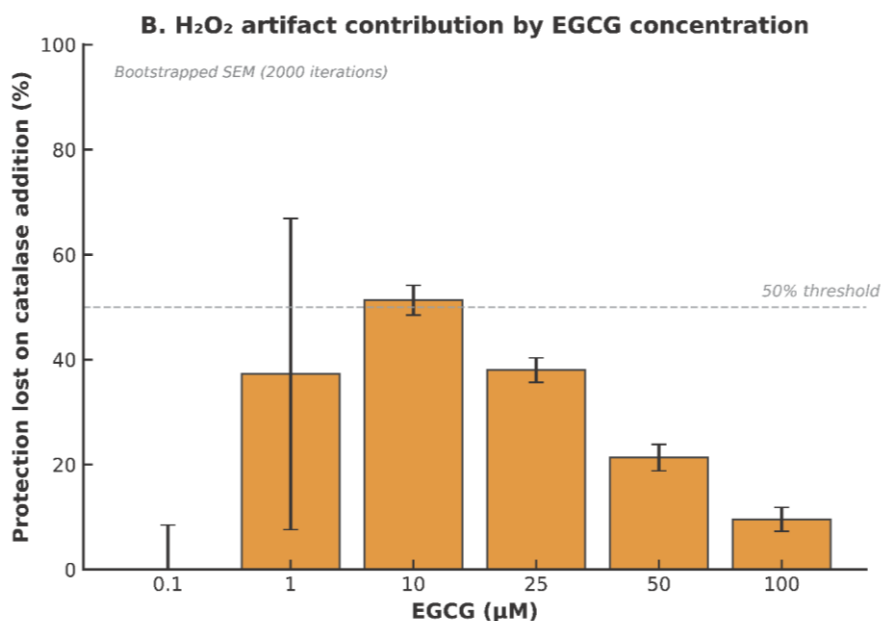


Figure 3. Catalase-sensitive fraction of apparent EGCG protection as a function of concentration. Bar plot of the percentage of apparent protection removed by catalase at each EGCG concentration, computed per biological replicate before averaging. The fraction declines from 51.3% at 10 μM to 9.6% at 100 μM. Error bars represent bootstrapped SEM ($n = 3$ biological replicates).

3.4 The protection that survives artifact control is concentration-graded and substantial

Protection in the artifact-controlled condition was not trivial at any tested concentration above 1 μM, and it followed a steep, monotonic dose-response through the 10–50 μM range (Figure 2), reaching its plateau by 50 μM and showing no further meaningful gain at 100 μM viability in Condition D moved from 53.1% at 10 μM to 79.2% at 50 μM but only to 90.2% at 100 μM, a pattern consistent with a saturating,

rather than linearly scaling, protective process. This indicates that, whatever the contribution of medium chemistry at low concentrations, EGCG retains a genuine, catalase-resistant protective action against MPP⁺ injury in this cellular system, and that this genuine action is itself concentration-dependent rather than constant across the tested range.

3.5 Reproducibility across biological replicates

Inter-replicate variability was modest throughout: coefficients of variation for

viability values stayed below 15% across the great majority of concentration-by-medium combinations, rising somewhat only at the lowest EGCG concentrations where absolute protection was small and a fixed amount of measurement noise therefore represented a larger fraction of the signal. The rank order of the four media conditions was preserved within every individual biological replicate, not only in the pooled average, and the direction of the catalase effect Condition D below Condition A at every EGCG concentration from 10 μ M upward was consistent across all three replicates without exception. This consistency supports treating the catalase-sensitive fraction as a stable, concentration-dependent property of the system rather than as an artifact of pooling noisy replicate data, and gives confidence that the gradient reported here would reproduce under independent replication using the same protocol.

Taken together, these results describe a single coherent pattern rather than five separate observations. The insult model is reproducible and well-characterised (§3.1); EGCG protects against it in every medium tested, but the size of that protection depends heavily on what the medium contains (§3.2); the dependency traces specifically to extracellular hydrogen peroxide, removable by catalase, whose contribution is large at low EGCG concentrations and shrinks steadily as concentration rises (§3.3); a genuine, catalase-resistant protective action persists throughout and itself follows an orderly concentration-response (§3.4); and the whole pattern holds consistently across independent biological replicates

rather than depending on any single experimental run (§3.5). The next section considers what this pattern means for how EGCG's cellular pharmacology should be read, and for how the wider literature on auto-oxidising polyphenols in cell culture might be re-read in its light.

4. DISCUSSION

This is the methodological contribution the field has been missing for a generation. Long and colleagues (2000) first showed that EGCG and related catechins generate micromolar hydrogen peroxide on contact with standard culture media; Long and Halliwell (2009) identified sodium pyruvate as the reason this generation goes undetected in pyruvate-containing formulations; and Halliwell has restated the underlying concern at intervals for more than a decade (Halliwell, 2003, 2007, 2008, 2014). What this body of work has lacked, until now, is a concentration-resolved figure for a disease-relevant cellular system a number those states plainly how much of a reported protection magnitude is medium chemistry and how much is the compound, at each concentration a reader might encounter in the literature.

The gradient reported here answers that question with more precision than the field has previously had available. Catalase removed 51.3% of apparent protection at 10 μ M EGCG, falling to 38.0% at 25 μ M, 21.3% at 50 μ M, and 9.6% at 100 μ M. Read plainly: at the low micromolar concentrations common throughout the historical EGCG-Parkinson's literature, something close to half of what a standard assay would record as neuroprotection is,

on this evidence, hydrogen-peroxide chemistry in the dish rather than pharmacology in the cell.

A semantic point is worth making explicit here, since it affects how the result should be read. The catalase-sensitive fraction is not equivalent to "false protection," and the language of artifact should not be taken to imply that the recorded viability gain is illusory. Cells genuinely survive better in the no-catalase condition; the open question is not whether that survival is real, but what causes it. A low dose of extracellular hydrogen peroxide can itself trigger a protective, hormetic stress response in surviving cells, distinct from any direct action of EGCG a possibility the present design cannot rule out and does not attempt to test. Alternatively, the peroxide may simply select against the most vulnerable cells in the population, producing a viability gain that reflects altered population composition rather than individual-cell rescue. Either mechanism is real cell biology, but neither is EGCG pharmacology in the sense the original protection claim implies, and conflating the two has likely inflated the apparent potency of EGCG, and plausibly of other auto-oxidising polyphenols, throughout this literature. Distinguishing between these two non-pharmacological explanations hormesis versus selective survival — would require single-cell tracking over the incubation period rather than the endpoint viability readout used here, and we flag it as an open question the present design was not built to resolve rather than one it has answered by omission.

The decline of the artifact's contribution with rising EGCG concentration is, in our reading, the most informative single feature of the dataset, because it offers a path to separating the two contributions rather than simply flagging that they are confounded. If medium-peroxide chemistry were the entire story, the catalase-sensitive fraction would stay roughly constant across the concentration range, since more EGCG should generate proportionally more peroxide. That it instead falls steeply, while overall protection continues to rise, indicates that a second, catalase-resistant mechanism becomes progressively more dominant as EGCG concentration increases. Characterising that mechanism was outside the scope of the present design; we examine it directly in a companion analysis using a chemical-to-cellular correlation framework across a benchmarked compound panel.

This has a direct, practical reading for the existing literature. Protection magnitudes reported in standard, pyruvate-containing medium at low micromolar EGCG concentrations a substantial fraction of the published EGCG-Parkinson's cell-culture literature should be treated as upper-bound estimates inflated by an uncontrolled, quantifiable artifact, rather than as clean measures of cellular pharmacology. The gradient reported here offers a starting point for applying that correction: a result obtained at 10 μM EGCG in uncontrolled medium might reasonably be discounted by roughly half before being compared with mechanistic claims; a result at 100 μM requires a far smaller correction.

We do not think the appropriate response to this finding is to discard the historical literature. Much of that work was careful within the experimental conventions of its time, and the auto-oxidation problem itself was, for much of the relevant period, known chemistry rather than common laboratory practice. The more useful response is forward-looking: artifact control should become a standard, rather than optional, feature of cellular polyphenol-protection studies. Any study testing EGCG, or a structurally related auto-oxidising polyphenol, in the low-to-mid micromolar range should include a pyruvate-free, catalase-supplemented medium arm of the kind used here, so that the hydrogen-peroxide-driven fraction of any observed protection is exposed rather than folded silently into the headline result. The additional cost is modest: catalase is inexpensive, pyruvate-free base medium is a standard catalogue item, and the extra condition doubles as a built-in artifact control rather than requiring a separate experiment.

Reporting conventions should follow the same logic. Manuscripts that report a cellular EGCG, or related polyphenol, protection magnitude should report the catalase-sensitive fraction alongside it wherever the medium and reagents allow; a protection number without that fraction is, on the present evidence, ambiguous, since it could reflect pharmacology, artifact, or some mixture of the two, and the reader has no way to judge which. Systematic reviews and meta-analyses of this literature should, in our view, classify studies that did not control for the artifact as a distinct, lower-confidence category,

rather than pooling them with artifact-controlled work as though the two measured the same quantity; pooling in that way inflates the apparent effect size and narrows the confidence interval around a number that is, in part, assay chemistry rather than biology.

The exclusion and analysis rules described in §2.7 were fixed before any cellular data were examined, and we report them in full, including the single replicate excluded under the pre-specified control-drift criterion, rather than describing only the final dataset. We raise this deliberately because the artifact problem addressed in this study and the broader problem of selective reporting are, in our view, related: both arise when a researcher has latitude to choose, after seeing the data, which numbers to feature and which controls to treat as optional. A protection magnitude obtained without a catalase-supplemented arm, and a protection magnitude obtained by quietly excluding an inconvenient replicate, fail for structurally similar reasons, and the same discipline deciding the rules before the result is known addresses both.

The four-condition design used here is straightforward enough to apply broadly, and we see its main value less in the specific numbers reported for EGCG against MPP⁺ in SH-SY5Y cells than in the general principle it embodies: any auto-oxidising compound tested in cell culture at concentrations where that auto-oxidation becomes chemically significant should be tested with and without a peroxide-clearing condition before its protective action is attributed to a specific cellular mechanism. EGCG is a useful case

study for this principle precisely because its auto-oxidation chemistry is unusually well characterised, but the principle extends to the wider class of polyphenols, several of which share EGCG's gallate or pyrogallol substructure and, with it, a comparable propensity to generate peroxide in culture.

Some limitations bear stating plainly. The present design used a single insult (MPP⁺) and a single cell line; whether the artifact gradient reported here generalises to 6-hydroxydopamine, to other dopaminergic models, or to other auto-oxidising polyphenols entirely is an empirical question this dataset cannot answer on its own, though the underlying chemistry (Akagawa et al., 2003; Yamamoto et al., 2004) gives reasonable grounds to expect a qualitatively similar pattern. The catalase-sensitive fraction also necessarily reflects extracellular peroxide specifically; it cannot speak to intracellular hydrogen peroxide generated by EGCG after cellular uptake, which catalase, unable to cross the plasma membrane, would not detect. Both limitations point toward useful extensions of the present design rather than undermining its central finding.

A final point concerns the broader pattern this finding sits within. EGCG is not the only polyphenol whose preclinical promise in neurodegenerative disease has been followed by clinical disappointment; resveratrol, curcumin, and several related compounds share both EGCG's chemical reactivity and, to varying degrees, its history of failing to translate cellular protection into clinical benefit. We do not claim that the auto-oxidation artifact quantified here explains those failures on

its own pharmacokinetic and blood-brain-barrier limitations almost certainly contribute as well, and lie outside what the present design can address. What this work does suggest is that part of the recurring gap between preclinical promise and clinical performance for chemically reactive polyphenols may trace, in some unknown proportion, to how the preclinical case was built rather than to the candidates themselves: a compound tested in conditions that do not control for its own reactivity will tend to look better in the dish than it later performs in the body, not because the underlying biology was misjudged, but because part of what was measured was never biology at all.

5. CONCLUSION

This study provides a concentration-resolved account of the hydrogen peroxide auto-oxidation artifact in EGCG cellular neuroprotection assays, using a dopaminergic, Parkinson's-relevant model. Catalase removed 51.3% of apparent EGCG protection at 10 μ M, falling to 38.0% at 25 μ M, 21.3% at 50 μ M, and 9.6% at 100 μ M, showing that the artifact's contribution is large at low concentrations and shrinks, rather than disappears, as EGCG concentration rises. Protection that survived artifact control was substantial at every concentration above 1 μ M, confirming that EGCG retains a genuine protective action against MPP⁺-induced dopaminergic injury once medium chemistry is accounted for.

These findings carry two practical consequences. First, protection magnitudes reported for EGCG, and plausibly for other auto-oxidising

polyphenols, in standard culture medium at low micromolar concentrations should be read with the present gradient in mind: a substantial share of such a result is likely to reflect medium chemistry rather than cellular pharmacology. Second, the four-condition media design used here offers a practical, low-cost addition to future cellular polyphenol-protection assays, requiring only a pyruvate-free base medium and commercially available catalase alongside the conditions a laboratory would already be running.

We see this contribution as recalibrating, rather than undermining, two decades of EGCG-Parkinson's cell-culture research. The qualitative finding that EGCG protects dopaminergic cells against MPP⁺ injury is not in question; what this work changes is the confidence with which a specific protection magnitude, reported under specific medium conditions, can be attributed to EGCG itself. A companion analysis extends this artifact-controlled framework to ask what mechanism accounts for the protection that remains once medium chemistry is removed from consideration.

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