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Article

Mitochondrial Function Under Combined Oral Contraceptive Exposure in a Neuroblastoma Model: A Preliminary Investigation

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Abstract

Background: Endogenous estradiol/progesterone (E2/P4) shape neuronal mitochondrial bioenergetics and redox. The mechanisms by which combined oral contraceptive (COC) steroids operate within physiologically relevant hormonal milieus remain poorly defined. **Methods:** Human SH-SY5Y cells (Cytion) were starved 48 h in Cytion medium without FBS and with 1% ITS, then exposed for 48 h to six conditions: vehicle (F0); follicular-like E2/P4 (F1); luteal-like E2/P4 (F2); F1 + dienogest/ethinylestradiol (DNG/EE; F3); F2 + DNG/EE (F4); and DNG/EE alone (F5). Primary endpoints were mitochondrial membrane potential (JC-1 red/green ratio) and ROS (H₂DCFDA/DCF); nitric oxide (DAF-FM) was also recorded. Hoechst 33342 nuclear fluorescence served both as a per-well proxy of cell number and as a proportionality factor for normalization. **Results:** Follicular- and luteal-like backgrounds produced distinct $\Delta\Psi_m$ /ROS set-points. Superimposing DNG/EE generated background-dependent shifts marked by higher DCF and lower JC-1 ratios versus vehicle; DAF-FM indicated concordant NO changes. Hoechst-based normalization preserved these patterns, indicating per-cell functional modulation rather than cell-number artifacts; proliferation differences were modest and background-dependent. **Conclusions:** Neuroendocrine background matters: physiologic E2/P4 milieus prime mitochondrial $\Delta\Psi_m$ /ROS/NO states in SH-SY5Y, onto which COC components impose context-dependent modulation at 48 h. These preliminary findings provide a standardized framework for subsequent image-based mitochondrial phenotyping.

Keywords: SH-SY5Y; mitochondrial membrane potential; JC-1; H₂DCFDA/DCF; estradiol; progesterone; dienogest; ethinylestradiol; combined oral contraceptives; neuroendocrinology; oxidative stress; mitochondrial dynamics

1. Introduction

Mitochondria are a principal interface between steroid signaling and neuronal bioenergetics/redox control. Endogenous estrogens modulate respiratory chain efficiency, antioxidant defenses, and mitochondrial membrane potential ($\Delta\Psi_m$) through both nuclear (ER α /ER β) and extranuclear signaling, including rapid actions that converge on mitochondrial proteins and biogenesis programs; in healthy neural contexts, these effects tend to favor resilience and efficient energy use—the “healthy-cell bias” of estrogenic action [1,2]. In parallel, progesterone and synthetic progestogens regulate mitochondrial function, calcium handling, and pro-survival pathways via classical and membrane-associated receptors, supporting neurotrophic and neuroprotective outcomes across diverse CNS models [3,4].

Against this backdrop, combined oral contraceptives (COCs)—typically ethinylestradiol (EE) plus a progestin (e.g., dienogest, DNG)—introduce exogenous steroids that can reshape systemic

redox balance and inflammatory tone. Multiple human studies report alterations in oxidative-stress biomarkers among COC users relative to non-users (e.g., hydroperoxides, antioxidant capacity, enzyme activities), though the direction and magnitude vary with formulation, dose, and physiology, highlighting the need for controlled cellular models to resolve mechanism and context [5-7]. In neuronal-like systems, where mitochondrial function is tightly coupled to viability and signaling, parsing how exogenous EE/progestins interact with physiologic hormonal milieus is particularly germane.

The SH-SY5Y human neuroblastoma line is a standard neuronal-like model to assay mitochondrial and oxidative endpoints, suitable for systematic interrogation of steroid effects under defined conditions [8,9]. For functional readouts, JC-1 yields a radiometric red/green signal proportional to $\Delta\Psi_m$, whereas H₂DCFDA/DCF provides a generic index of intracellular ROS; both are widely adopted when implemented with appropriate controls and awareness of probe caveats (e.g., dye loading/efflux, oxidation chemistry, and the necessity of orthogonal validation) [10-12]. Beyond plate-reader assays, Mitotracker imaging coupled with quantitative morphometry (e.g., area, aspect ratio, form factor, perinuclear density) captures structural correlates of mitochondrial function, and QuPath provides an open, reproducible framework for high-throughput feature extraction and downstream statistics [13-15].

Physiological follicular and luteal milieus differ chiefly in progesterone (low vs. high) with estradiol spanning lower-to-mid ranges across sub-phases; these fluctuations plausibly set distinct mitochondrial “set-points” in neural cells [16,17]. Emulating such backgrounds in vitro offers a principled way to test context-dependent actions of EE and progestins. Here we use SH-SY5Y cells to (i) establish follicular-like and luteal-like conditions with physiologically inspired E2/P4 combinations; (ii) overlay DNG/EE on each background and as a standalone exposure; and (iii) quantify $\Delta\Psi_m$ (JC-1) and ROS (DCF) after 48 h, a window chosen to capture stabilized, transcriptionally integrated bioenergetic responses beyond acute dye or ion-flux artifacts. Within this framework, our expectation is that the E2/P4 milieu defines baseline $\Delta\Psi_m$ /ROS states and that DNG/EE imposes background-dependent shifts at 48 h—directionality that we will subsequently connect to Mitotracker-based morphometry (via QuPath) to bridge functional readouts with structural phenotypes.

2. Materials and Methods

2.1. Cell line and Culture Conditions

Cells. SH-SY5Y human neuroblastoma cells (Cytion; catalog/lot per supplier documentation) were maintained according to the Cytion technical datasheet.

Incubation. 37 °C, 5% CO₂, saturated humidity.

Starvation prior to treatment. 48 h in Cytion medium without FBS, supplemented with 1% ITS (insulin–transferrin–selenium), to minimize exogenous steroid interference and synchronize metabolic state. Cells were then exposed to test conditions for 48 h.

2.2. Experimental Design and Treatment Conditions

Six conditions were tested (F0–F5), emulating follicular-like and luteal-like hormonal milieus, with or without combined oral contraceptive (COC) steroids.

Table 1. Nominal compositions (per well).

Condition	Progesterone (P4) [nM]*	Estradiol (E2) [nM]*	Dienogest (DNG) [nM]*	Ethinylestradiol (EE) [nM]*	Description
F0	0	0	0	0	Vehicle (control)

F1	1.87	0.28	0	0	Follicular-like background
F2	40.0	0.50	0	0	Luteal-like background
F3	1.87	0.28	160	0.30	F1 + DNG/EE
F4	40.0	0.50	160	0.30	F2 + DNG/EE
F5	0	0	160	0.30	DNG/EE only

Final DMSO ≤ 0.1% v/v in all wells.

2.3. Plate Layout and Controls

Experiments were run on multi-well plates (96-well unless otherwise noted) following the “Piastre” map in the spreadsheet. Each condition included n = 8 technical replicates per plate. Controls included:

- Vehicle (F0; “CTR”), on every plate.
- Positive ROS control: H₂O₂ 100 μM for 60 min (plate-end spike) where indicated.
- Antioxidant control: N-acetyl-L-cysteine (NAC) 0.5 mM maintained throughout the 48 h exposure (where indicated).
- ΔΨm depolarization control (for JC-1): CCCP/FCCP 50 μM for 5 min before reading; plates kept protected from light.

2.4. Fluorimetric Assays

All probes were handled under low light; identical exposure/gain settings were used across conditions on the same plate.

2.5. JC-1 (Mitochondrial Membrane Potential, ΔΨm)

Cells were washed with PBS and incubated with JC-1 at the manufacturer-recommended working concentration for 20–30 min at 37 °C. Fluorescence was read at:

- Green (monomer): Ex 485–490 nm / Em ~530 nm
- Red (aggregate): Ex 525–540 nm / Em 590–610 nm
- The red/green ratio was used as the primary ΔΨm endpoint.

2.6. H₂DCFDA/DCF (ROS)

Cells were incubated with H₂DCFDA (typical 10–20 μM) for 30 min at 37 °C, washed, and read at Ex 485–495 nm / Em 525–535 nm. Where plate-end H₂O₂ spikes were used, readings were taken immediately after the 60 min incubation.

2.7. DAF-FM Diacetate (Nitric Oxide, NO)

After 48 h treatment, wells were washed and incubated with DAF-FM DA (per supplier’s instructions) for 30–45 min at 37 °C, followed by a de-esterification period in probe-free buffer (15–30 min). Fluorescence was read at Ex ~495 nm / Em ~515 nm.

2.8. Hoechst 33342 (Nuclear Content/Proportionality)

At the end of the 48 h exposure, nuclei were stained with Hoechst 33342 (0.5–5 μg/mL) for 10–15 min at 37 °C, and read at Ex ~350–360 nm / Em ~460–470 nm. Integrated Hoechst signal per well was used both as a proxy for cell number/proliferation at 48 h and as a proportionality factor for normalization of functional readouts (DCF, JC-1 ratio).

2.9. Data Processing and Normalization

Raw plate data were exported to MATLAB for analysis. For each well:

- Primary endpoints: JC-1 red/green ratio ($\Delta\Psi_m$), DCF (ROS), DAF-FM (NO), and Hoechst (nuclear signal).
- Normalization: In addition to raw values, we report Hoechst-normalized endpoints to correct for cell-number bias:

$$\text{Endpoint}_{\text{norm}} = \frac{\text{Endpoint (DCF or JC-1 ratio)}}{\text{Hoechst integrated fluorescence}}$$

- Aggregation: Technical replicates ($n = 8$) were summarized per condition/plate before inferential testing.

2.10. Quality Control and Statistics

Data underwent visual QC and robust outlier handling (pre-specified rules; details/code in Supplementary). Unless distributions justified parametric tests, we used non-parametric approaches:

- Across-condition comparisons: Kruskal–Wallis.
- Pairwise contrasts (e.g., F1–F5 vs F0; F1 vs F2; background effects F3 vs F1, F4 vs F2): Mann–Whitney tests, with Benjamini–Hochberg FDR control ($q = 0.05$).
- Effect sizes: median differences and rank-biserial correlation; 95% CIs by bootstrap where applicable.
- All analyses were run in MATLAB (The MathWorks, Natick MA) with custom scripts (provided as Supplementary)

2.11. Reagents and Solutions

P4, E2, DNG, and EE were cell-culture grade; stocks were prepared in DMSO and diluted in medium to reach the final [units] indicated in the table (final DMSO $\leq 0.1\%$ v/v). Probes: JC-1, H₂DCFDA, DAF-FM DA, and Hoechst 33342 (supplier and catalogue numbers to be listed in the Reagents subsection or Supplementary Table).

3. Results

3.1. Data Quality, Replicates, and Normalization

Across plates, each condition (F0–F5) included $n = 8$ technical replicates. After the pre-registered robust QC/outlier handling, effective replicate numbers per condition remained adequate for non-parametric inference (median $n_{\text{clean}} \approx 5$; range 5–8). Hoechst 33342 integrated fluorescence at 48 h was used both as a proxy of cell number/proliferation and as a proportionality factor for functional readouts (DCF; JC-1 ratio). Normalization to Hoechst did not invert qualitative patterns observed in raw signals.

3.2. Proliferation (Hoechst 33342)

Hoechst fluorescence indicated modest, background-dependent differences in cell number at 48 h. Relative to vehicle (F0), follicular-like (F1) and luteal-like (F2) milieus showed only small changes in Hoechst signal; adding DNG/EE (F3, F4) or using DNG/EE alone (F5) did not produce large proliferation shifts. Consequently, Hoechst-normalized endpoints closely tracked raw effects, suggesting per-cell functional modulation rather than artifacts from varying cell numbers (Figure S1; Table S1).

3.3. ROS (DCF)

DCF differed significantly across conditions (Kruskal–Wallis, FDR-controlled). Versus F0, F1–F5 showed higher DCF, with the largest increase in F3 (F1+ DNG/EE) and a marked elevation also in F4 (F2+ DNG/EE). F5 (DNG/EE alone) was elevated vs F0 but lower than phase-plus-COC conditions. These contrasts remained significant after Hoechst normalization (pairwise Mann–Whitney, FDR; Figure 1A).

3.4. Mitochondrial Membrane Potential (JC-1 Ratio)

The JC-1 red/green ratio showed a significant across-group effect (Kruskal–Wallis, FDR). Relative to F0, F1–F5 exhibited a decrease in JC-1 ratio ($\Delta\Psi_m$ depolarization), most pronounced in F4 (luteal-like + DNG/EE) and substantial in F3 (follicular-like + DNG/EE). F5 showed a smaller but consistent decrease. Effects persisted after Hoechst normalization (pairwise Mann–Whitney, FDR; Figure 1B).

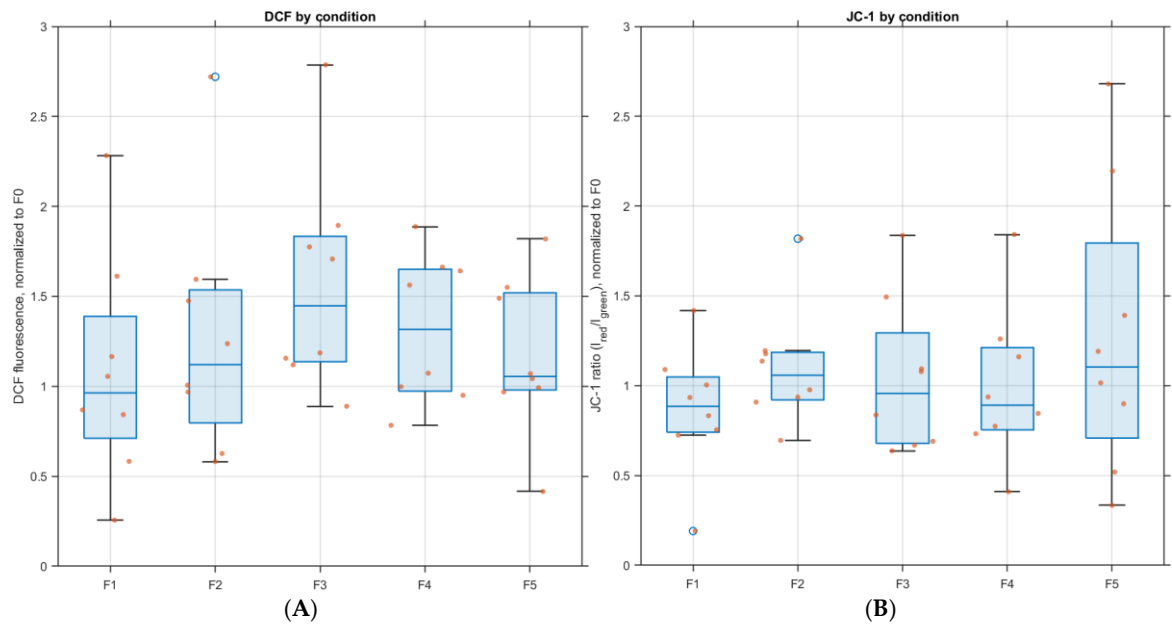


Figure 1. Distribution of JC-1 ratio (A) and DCF fluorescence (B) across experimental conditions. Individual points represent biological replicates (n = 8 per condition). Boxplots indicate median and interquartile range; whiskers extend to 1.5× IQR.

3.5. Nitric Oxide (DAF-FM)

DAF-FM did not differ significantly across conditions (Kruskal–Wallis, n.s.), and no pairwise comparison vs F0 survived FDR correction. Thus, within 48 h, NO changes were not robust in this model under the tested exposures.

3.6. Background-Dependence (Follicular vs Luteal Milieus)

Comparisons of F3 vs F1 and F4 vs F2 support a background-dependent modulation by DNG/EE:

- ROS (DCF): the largest ROS rise occurred on the follicular-like background (F3 > F4).
- $\Delta\Psi_m$ (JC-1): the strongest depolarization occurred on the luteal-like background (F4 > F3).
- A simple interaction contrast (F4–F2) vs (F3–F1) was directional for both endpoints, indicating that phase-like hormonal context primes mitochondrial responses to COC steroids at 48 h (Figure 2 A and B).

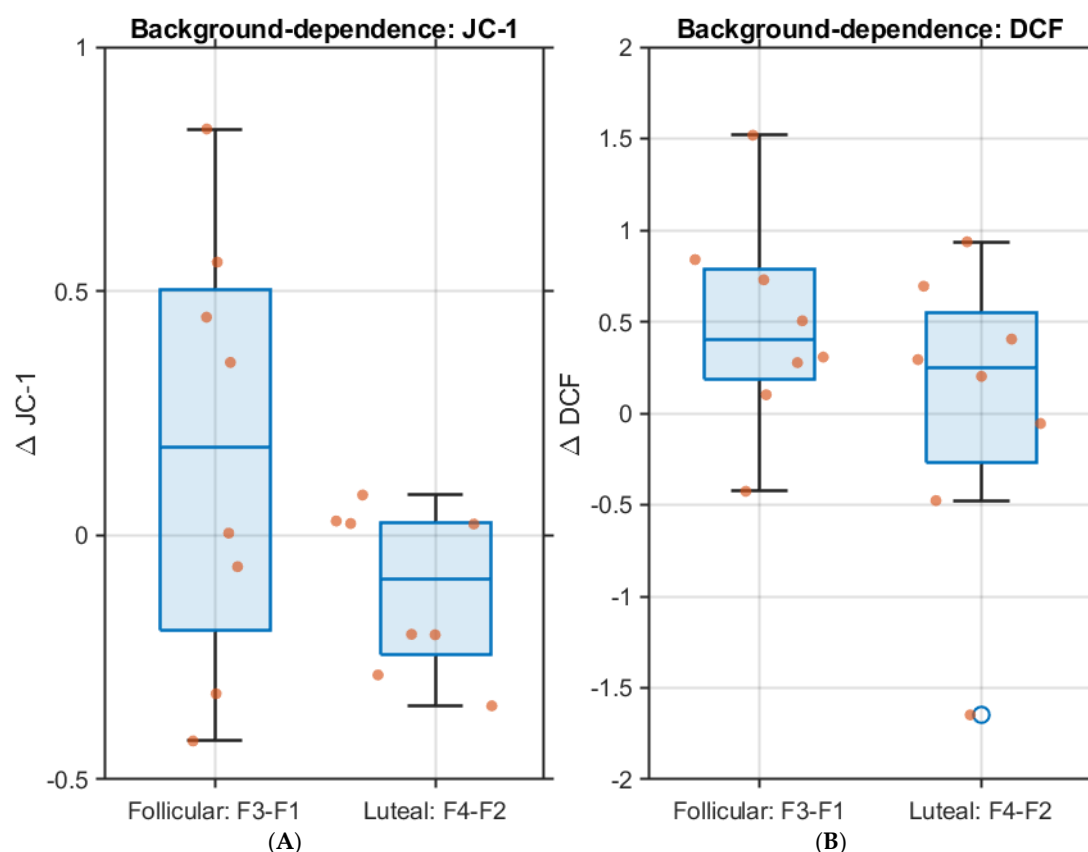


Figure 2. Background-dependent effects of DNG/EE on JC-1 ratio (A) and DCF fluorescence (B). Differences were calculated for paired biological replicates as F3 – F1 (follicular-like background) and F4 – F2 (luteal-like background). Individual points represent biological replicates; boxplots show median and interquartile range.

3.7. Sensitivity Analyses and Controls

Sensitivity analyses (with/without Hoechst normalization; with/without aggressive outlier trimming) did not change the direction of main effects. Where included, positive/negative controls behaved as expected (e.g., H_2O_2 increased DCF; CCCP/FCCP decreased JC-1 ratio), supporting assay validity.

4. Discussion

This preliminary study underscores the importance of neuro-endocrine background in shaping mitochondrial readouts in neuronal-like cells. Using SH-SY5Y cells exposed for 48 h to phase-inspired E2/P4 milieus with or without dienogest/ethinylestradiol (DNG/EE), we observed two consistent functional outcomes: (i) a significant rise in DCF (ROS) and (ii) a decrease in JC-1 red/green ratio ($\Delta\Psi m$ depolarization) relative to vehicle. These effects persisted even after Hoechst proportionality correction, indicating that changes reflect per-cell modulation rather than differences in cell number/proliferation at 48 h. By contrast, no significant differences emerged for DAF-FM (NO) following multiple-testing correction. The absence of NO changes can be interpreted in light of the fast, transient dynamics of nitric oxide and the well-documented methodological limitations of DAF probes (e.g., oxidant cross-reactivity and esterase dependence), which reduce sensitivity to subtle condition-specific fluctuations [18-20].

The hormonal phase-like backgrounds modulated the magnitude and the direction of the responses. Adding DNG/EE on a follicular-like milieu (F3) produced the strongest ROS increase, whereas the luteal-like milieu with DNG/EE (F4) showed the most pronounced $\Delta\Psi m$ depolarization. These observations suggest that the E2/P4 set-point primes mitochondrial susceptibility, a concept consistent with growing evidence that sex steroids regulate mitochondrial bioenergetics, redox homeostasis, and vulnerability to metabolic stress [21-28]. Lower P4 in the follicular-like context may

permissively amplify ROS, while higher P4 in the luteal-like condition may interact with progesterin/estrogen signaling to bias $\Delta\Psi_m$ shifts [29,30]. Although receptor engagement was not directly measured, the background-dependent outcomes align with modern models of steroid signaling that include ER/PR/GPER/mPR and direct mitochondrial receptor targets [21,31]. The dynamic role of sex hormones in neuronal mitochondrial function—including regulation of OXPHOS, antioxidant systems, and biogenesis—supports this interpretation [25-28,32,33].

Our assay validation strengthens confidence in these findings: H_2O_2 increased DCF as expected, and CCCP/FCCP decreased the JC-1 ratio, confirming mitochondrial sensitivity. Importantly, Hoechst-based additional proportionality affirmed that functional effects were not artifacts of altered cell number.

Still, several limitations need to be acknowledged: SH-SY5Y cells although neuron-like, do not exhibit the full complexity of mature neurons. We used a single exposure window (48 h); JC-1 and DCF have methodological constraints regarding specificity and quantitiveness (Yin et al., 2021). Likewise, we did not include orthogonal respiration or NO assays. These boundaries motivate the next experimental phase, which will incorporate Mitotracker-based morphometry (QuPath) to test structural correlates of $\Delta\Psi_m$ /ROS trends, complemented—if desired—by orthogonal functional endpoints (e.g., TMRE/TMRM for $\Delta\Psi_m$, MitoSOX/Amplex assays for ROS subtypes) and time-course sampling to capture early NO transients.

The joint analysis of JC-1 and DCF signals further supports the notion that oxidative stress and mitochondrial polarization represent partially independent outputs of the cellular response to the hormonal environment. As shown in Figure 3, similar JC-1 ratios can be associated with different levels of DCF fluorescence depending on the experimental condition, suggesting that the progestinic index modulates redox balance beyond its effects on mitochondrial membrane potential.

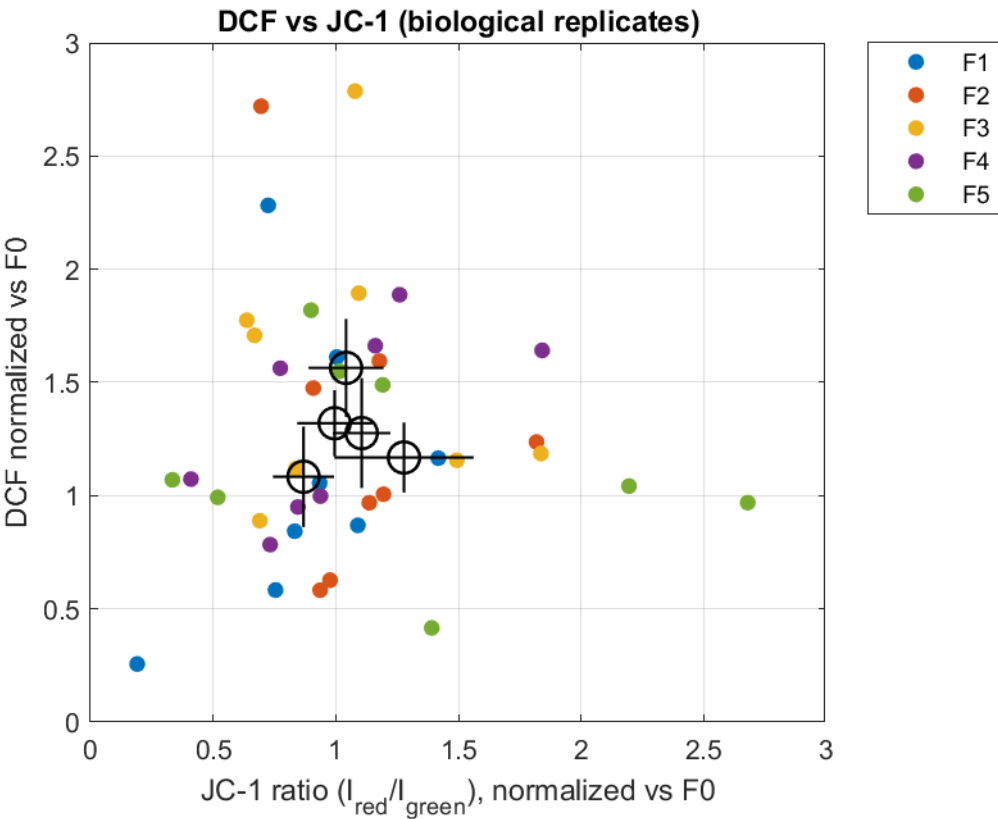


Figure 3. Relationship between mitochondrial polarization (JC-1 ratio) and oxidative stress (DCF fluorescence). Individual points represent biological replicates, colored by experimental condition. Larger open symbols indicate condition means $\pm$ SEM, highlighting condition-specific relationships between the two readouts.

Overall, our findings support a framework in which physiological hormonal milieus (follicular vs luteal) establish distinct mitochondrial set-points, and COC-like steroids superimpose context-dependent alterations in ROS and $\Delta\Psi_m$ at 48 h. This aligns with modern neuroendocrine-mitochondrial models positing that hormonal status is not a passive backdrop but an active determinant of mitochondrial responsiveness [21,25-29]. This integrated view (hormones $\times$ mitochondria $\times$ receptor signaling) has implications for understanding how menstrual cycle phase, hormonal therapies, and contraceptives may modulate neuronal resilience, redox state, and susceptibility to metabolic or oxidative stress.

From a translational perspective, these findings raise relevant questions: Do fluctuating steroid levels during the menstrual cycle alter neuronal mitochondrial vulnerability? Could long-term exposure to combined oral contraceptives reshape neuronal mitochondrial baseline states? Such questions require validation in more physiological systems—including primary neurons, brain organoids, and in vivo models—as well as functional endpoints involving neuronal survival, synaptic signaling, and plasticity. Recent work underscores how sex hormones influence CNS energy metabolism and oxidative pathways [29,30], supporting the relevance of hormonal context in mitochondrial research.

5. Conclusions

In conclusion, our findings show that E2/P4 hormonal milieus prime the mitochondrial state of SH-SY5Y cells, with follicular- and luteal-like backgrounds establishing distinct $\Delta\Psi_m$ and ROS set-points at 48 h. Within these contexts, COC components exert background-dependent effects, as DNG/EE most strongly enhance ROS under follicular-like conditions while inducing the greatest $\Delta\Psi_m$ depolarization in a luteal-like milieu. These outcomes reflect true per-cell changes rather than artifacts of cell number, given the preserved Hoechst proportionality of DCF/JC-1 signals and the minimal differences in proliferation. Conversely, NO levels did not show robust modulation at 48 h, as DAF-FM measurements revealed no significant between-group differences after FDR correction. Together, these results provide a rationale for follow-up image-based Mitotracker morphometry (QuPath) and complementary functional assays to link mitochondrial structural features with the observed shifts in $\Delta\Psi_m$ and ROS.

Taken together, our results highlight the importance of the neuroendocrine context when evaluating the mitochondrial impact of combined oral-contraceptive steroids in neuronal-like cells and provide a standardized foundation for deeper mechanistic analyses.

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