

Selenium supplementation protects trophoblast cells from oxidative stress

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1 **Final Draft**

2

3 **Selenium Supplementation Protects Trophoblast Cells from**
4 **Mitochondrial Oxidative Stress.**

5

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23 **Keywords: Trophoblast, Oxidative Stress, Selenium, Reactive Oxygen Species,**
24 **Antioxidants**

25

26 **ABSTRACT**

27 **INTRODUCTION:** Oxidative stress plays an important role in the pathogenesis of
28 preeclampsia, a placental disorder affecting approximately 7% of pregnancies.
29 Trophoblast cells are susceptible to oxidative stress which causes increased cell
30 death and placental turnover. In this study, inhibitors of the mitochondrial respiratory
31 chain were utilised to induce oxidative stress and the effect that selenium
32 supplementation had on trophoblast viability was investigated.

33 **METHODS:** Trophoblast cells (BeWo, JEG-3 and Swan-71) were treated with Na
34 Selenite (100nM) or Selenomethionine (500nM) to increase the biological activity of
35 antioxidants Glutathione Peroxidase and Thioredoxin Reductase. The cells were
36 then oxidatively stressed with the addition of increasing doses of Antimycin C and
37 Rotenone and the Resazurin end point assay was used to assess cellular activity.

38 **RESULTS:** There was a significant dose dependent decrease in the cellular activity
39 in BeWo, JEG-3 and Swan-71 when treated for 4 hours with increasing
40 concentrations of Antimycin (40-320 μ M) and Rotenone (100-800nM). Prior
41 Incubation with Na Selenite and Selenomethionine was able to protect trophoblast
42 cells from oxidative stress at Rotenone concentrations of 200nM and 400nM
43 ($P < 0.001$) and Antimycin concentrations of 80-240 μ M ($P < 0.001$).

44 **DISCUSSION:** These data suggest that selenoproteins such as Glutathione
45 Peroxidase and Thioredoxin Reductase have an important role in protecting
46 trophoblast mitochondria from oxidative stress.

47 **CONCLUSIONS:** This study emphasises the importance of maintaining an adequate
48 selenium supply during pregnancy and especially in pregnancies complicated by
49 conditions such as preeclampsia.

50

51

52 INTRODUCTION

53 Oxidative stress describes a condition where the presence of reactive oxygen and
54 nitrogen species (RONS) overwhelms cellular antioxidant defences. The initial
55 response to oxidative stress is to up regulate defensive mechanisms such as heat
56 shock proteins and anti-oxidant enzymes. Cells enter a state of suspended
57 animation whilst the stress is counteracted or disperses at which point the cells
58 return to homeostasis. If the oxidative insult persists, oxidised macromolecules such
59 as lipids, proteins and nucleic acids are released. Persistent or excessive oxidative
60 stress will lead to autophagy, apoptosis and necrosis with the associated release of
61 cellular debris [extensively reviewed 1]. Placental oxidative stress is central in the
62 pathogenesis of many disorders of pregnancy including preeclampsia and preterm
63 labour and numerous studies have shown that there is increased shedding of
64 oxidised macromolecules as well as cellular debris from placentae affected by these
65 disorders [2,3].

66 Anti-oxidant enzymes are central to protecting cells from oxidative stress. These
67 include enzymes involved in the Glutathione and Thioredoxin reducing systems such
68 as Glutathione Peroxidase (GPx) and Thioredoxin Reductase (ThxRed). These two
69 proteins are selenoenzymes, whereby activity in all cells and tissues is dependent
70 upon an adequate supply of the essential micronutrient selenium. This provides an
71 elegant mechanism for up-regulation, as selenium supplementation is able to
72 promote the activity of these proteins in many cell types, in animal studies and in
73 humans [4,5,6].

74 Previous work from this laboratory proposed that the up-regulation of GPx and
75 ThxRed using selenium supplementation would protect cardiac tissues from the
76 damaging effects of the oxidative stress during clinical procedures including coronary
77 artery bypass grafts. This proved to be the case and in clinical studies the beneficial
78 effects of selenium supplementation, in association with other essential
79 micronutrients, resulted in significantly quicker patient recoveries [7]. The same
80 approach is an attractive proposition when considering placental oxidative stress in
81 complications of pregnancy such as preeclampsia. Could maximising placental
82 expression of these proteins using selenium supplementation protect trophoblast
83 from oxidative stress?

84 In a recently published paper we demonstrated that up-regulation of GPx and
85 ThxRed using both inorganic sodium selenite and organic selenomethionine was
86 able to protect trophoblast from exogenously added reactive oxygen species such as
87 peroxides [8]. In the present study we have asked the same question but generated
88 the oxidative stress endogenously by selectively blocking the electron transport
89 chain and generating mitochondrial oxidative stress.

90 **METHODS AND MATERIALS.**

91 ***Cell Culture and Reagents:***

92 The human choriocarcinoma cell lines BeWo and JEG-3 cells were obtained from
93 American Tissue Culture Collection (ATCC). The non-choriocarcinomic trophoblastic
94 cell line Swan-71 was kindly provided by Professor Gil Mor, Yale University. Cells
95 were grown and maintained at 37°C with 5% CO₂ in Dulbecco's Modified Eagle
96 Medium (Invitrogen, Australia) containing 10% fetal bovine serum and 500 U/mL
97 penicillin–streptomycin (Invitrogen, Australia). Antimycin and Rotenone were
98 obtained from Sigma-Aldrich Ltd, Australia. All other reagents, unless stated
99 otherwise, were obtained from Sigma- Aldrich Ltd, Australia. Trophoblast cells were
100 collected from 75cc flasks by trypsinization and were seeded in 96 well sterile tissue
101 culture plates at 10,000 cells per well for treatment and analysis.

102

103 ***Cell Extraction:***

104 To extract protein for biochemical analysis, the confluent monolayer from 75cc flasks
105 was collected after 24 hours of continuous culture in selenium supplemented media
106 at 100nM NaSe and 500nM SeMet. The collected cell pellet was resuspended in
107 300µL of cell lysis reagent (50mM Tris-HCl, 150mM NaCl, 1% Triton and 1% Tween-
108 20) and incubated on ice for 30 min. The lysed cell suspension was centrifuged at
109 12000 rpm for 5 min to pellet cell debris and the protein-containing supernatant was
110 removed and stored at -20°C until use.

111

112 ***Protein Estimations:***

113 Quantification of the protein content from the cell extracts was performed using a
114 BCA Protein Assay kit (Pierce, Rockford, USA) following the manufacturers protocol.
115 Bovine serum albumin (BSA) was used as the protein standard, enabling the

116 construction of a standard curve, from which unknown protein concentrations could
117 be extrapolated. Protein concentrations are expressed as mg of protein per mL of
118 cell extract (mg/mL).

119 ***Glutathione Peroxidase (GPx) Assay:***

120 Glutathione Peroxidase activity was quantified using a GPx assay, as first described
121 by Flohe & Gunzler [9]. Glutathione Peroxidase present in the protein cell extract
122 reduces the hydroperoxide substrate tert-butyl hydrogen peroxide and becomes
123 oxidised. The oxidised GPx is regenerated by glutathione (GSH), which becomes
124 oxidised to glutathione disulphide (GSSG). The GSSG can then be returned to GSH
125 by the donation of two electrons from NADPH via the enzyme glutathione reductase
126 (GR). The rate of decrease of NADPH directly correlates to GPx activity in the cell
127 extract and can be measured spectrophotometrically at 340nm using a Tecan
128 Sunrise plate reader with Magellan software. The activity measured was expressed
129 as milliunits per milligram of protein (mU/mg of protein). The inter assay and intra
130 assay coefficients of variation were 4.43% and 7.96% respectively.

131 ***Thioredoxin Reductase (ThxRed) Assay:***

132 A commercial Thioredoxin Reductase assay was used to determine the activity in
133 cells extracts. The kit was purchased from Cayman Chemical Company and 20 μ L of
134 cell extract was assayed in triplicate. The results were expressed as nmols activity
135 per mg protein. The inter assay and intra assay coefficients of variation were 7.47%
136 and 7.19% respectively

137

138 ***Selenium Supplementation and oxidative stress:***

139 Trophoblast cell number was adjusted to 100,000 cells per mL and 100 μ L was
140 added to each well in a 96 well plate and incubated for 24 h at 37°C. Cells were then
141 treated with selenium in the form of inorganic sodium selenite (NaSe) or organic
142 selenium methionine (SeMet) prepared in media and added at concentrations of 100
143 nM or 500nM respectively. Cells were further incubated at 37°C for 24h. Trophoblast
144 cells were then oxidatively stressed with various concentrations of Antimycin (40,
145 80,160, 240, 340 μ M) and Rotenone (100, 200, 400, 600, 800 nM) and incubated for
146 4 h at 37°C with 5% CO₂.

147

148 **Resazurin End Point Assay:**

149 To assess cellular function the Resazurin end point assay was used. This assay is
150 an oxidation-reduction indicator, detecting the production of NADH/NADPH and
151 assessing the mitochondrial metabolic activity [10]. Resazurin is non-fluorescent until
152 it is reduced to resorfin, which is highly fluorescent. Following treatments as
153 described above the media was removed by vacuum aspiration and cells were
154 washed twice with DPBS. Resazurin (200ul of 40nM) was added to each well
155 followed by a 2 hr incubation. The degree of reduction of resazurin to resorufin was
156 determined by fluorescence (excitation 530 nm; emission 590 nm) using a
157 Fluoroskan Ascent microplate fluorometer (Thermo Scientific, Australia).

158

159 **Statistical Analysis**

160

161 Statistical analysis was performed using Graph Pad, PRISM version 5.03 for
162 Windows, GraphPad Software, San Diego California USA, www.graphpad.com.
163 OneWay analysis of variance (ANOVA) with Tukey's Post Hoc testing was used to
164 analyse significant differences between selenium and non-selenium treatment
165 groups of equal n value for all treatment regimes. P values less than 0.05 were
166 considered significant and all data is presented as mean +/- standard deviation.
167 Each experiment was repeated a minimum of three times with all experiments
168 conducted in triplicate.

169

170 **Results**

171 ***Glutathione Peroxidase (GPx) and Thioredoxin Reductase (ThxRed) activity***
172 ***post selenium supplementation***

173

174 In BeWo cells, the baseline level of GPx activity was 129.6 ± 15.17 mU/mg (Fig 1).
175 After the supplementation with NaSe (100nM) or SeMet (500 nM), GPx activity was
176 increased to levels of 283.5 ± 14.24 mU/mg and 273.7 ± 14.49 mU/mg of protein. In
177 the BeWo cell line ThxRed baseline activity was 18.53 ± 2.11 mU/mg of protein. A
178 significant increase ($P < 0.05$) in ThxRed activity was measured for cultures treated
179 with 100nM NaSe (72.14 ± 7.34 mU/mg of protein) and 500nM SeMet (69.52 ± 5.83
180 mU/mg of protein). Similar significant increases in GPx and ThxRed activity were

181 seen in JEG-3 cells, however, although the Swan-71 cells showed an increase level
182 of activity post Se supplementation, it did not reach significance.

183

184 ***Selenium Supplementation Protects from Oxidative Stress induced by***
185 ***Rotenone treatment.***

186

187 BeWo, JEG-3 and Swan-71 cell lines were cultured in either media only or
188 supplemented with 100 nM NaSe or 500 nM SeMet to induce GPx and ThxRed
189 activity. Cells were subsequently stressed with increasing doses of Rotenone (100,
190 200, 400, 800 nM) and cellular activity determined using the reszaurin end point
191 assay (Fig 2). Significant reductions in cellular activity were observed in cells
192 exposed to all rotenone concentrations ($P < 0.001$). Prior incubation with NaSe was
193 demonstrated to protect trophoblast cells from oxidative stress at concentrations of
194 400 nM ($70.99\% \pm 24.26$; $P < 0.001$) and 200 nM ($88.56\% \pm 18.81$; $P < 0.05$) when
195 compared to unsupplemented control cultures that displayed cellular activities of
196 $36.77\% \pm 20.19$ (400nM) and $71.72\% \pm 26.03$ (200nM) respectively. Similarly, prior
197 treatment with 500nM SeMet resulted in protection from rotenone-induced stress at
198 concentrations of 400nM ($66.92\% \pm 26.20$; $P < 0.001$) and 200nM ($101.3\% \pm 15.96$;
199 $P < 0.05$). Identical observations were made with JEG-3 and Swan-71 cells.

200 ***Selenium Supplementation Protection from Oxidative Stress induced by***
201 ***Antimycin treatment.***

202 BeWo, JEG-3 and Swan-71 cell lines were cultured in either media or supplemented
203 with 100 nM NaSe or 500 nM SeMet and subsequently stressed with increasing
204 doses of Antimycin. Antimycin concentrations of $80\mu\text{M}$ to $320\mu\text{M}$, applied for 4 hours,
205 resulted in a dose dependent significant decrease in cellular activity in all three cell
206 types ($P < 0.0001$). As shown in Figure 3a, Swan-71 cells supplemented with NaSe
207 exhibited protection from oxidative stress at Antimycin concentrations of $80\mu\text{M}$
208 ($86.26\% \pm 16.45$ vs. $52.60\% \pm 23.57$; $P < 0.001$), $120\mu\text{M}$ ($75.06\% \pm 9.47$ vs. 47.03%
209 ± 12.09 ; $P < 0.01$), $160\mu\text{M}$ ($78.16\% \pm 21.72$ vs. $26.74\% \pm 21.86$; $P < 0.001$) and $240\mu\text{M}$
210 ($53.74\% \pm 22.88$ vs. $11.51\% \pm 9.96$; $P < 0.001$). Prior treatment with SeMet also
211 resulted in protection from Antimycin induced oxidative stress when exposed to
212 concentrations of $80\mu\text{M}$ ($P < 0.01$), $120\mu\text{M}$ ($P < 0.01$), $160\mu\text{M}$ ($P < 0.001$) and $240\mu\text{M}$
213 ($P < 0.001$) (Figure 3b). For BeWo and Jeg-3 cell lines, protection against Antimycin

214 induced oxidative stress was observed only for the 320µM-160µM concentration
215 range (P<0.001).

216 **DISCUSSION**

217 All aerobic tissues generate reactive oxygen and nitrogen species (RONS) and
218 ample anti-oxidant production is essential for normal homeostasis. Oxidative stress
219 arises when there is an excessive production of (RONS) or there is a diminished
220 capacity of anti-oxidants to negate the detrimental effects of RONS. Typically a
221 tissue experiencing oxidative stress will release evidence of this stress as oxidised
222 lipids, proteins and nucleic acids. Excessive oxidative stress will result in tissue
223 turnover and release of cellular components through apoptotic and necrotic
224 mechanisms [1]. There are numerous reports on the release of oxidised
225 macromolecules from the human placenta and oxidative stress has been proposed
226 as a driver of cellular turnover in this tissue, especially during pathologies such as
227 preeclampsia [11,12].

228

229 Glutathione peroxidase (GPx) and Thioredoxin reductase (ThxRed) are seleno-
230 proteins and critical components of two very important anti-oxidant systems.
231 Diminished GPx and ThxRed activity results in the generation of oxidative stress
232 demonstrating a causative effect. Furthermore, there is decreased activity of these
233 enzymes in tissues as a consequence of oxidative stress [4,7,11]. As seleno-proteins
234 the expression and activity of GPx and ThxRed is dependent upon an adequate
235 supply of selenium and the level of expression in cells and animal models can be
236 controlled by selenium supplementation. Several reports have suggested that
237 preeclamptic mothers are seleno-deficient and selenium intake may be important in
238 the development of preeclampsia [4,13,14]. Hence, the purpose of this study was to
239 investigate the link between selenium supplementation and protecting placental cells
240 from oxidative stress.

241

242 Two forms of selenium, organic SeMet and inorganic NaSe were used to up regulate
243 GPx and ThxRed in the trophoblastic cell lines BeWo, JEG-3 and Swan-71. Enzyme
244 assays for GPx and ThxRed were used to confirm the up-regulation of activity post-
245 supplementation. Similar observations have been made in many other cell types and
246 in animal models [15,16]. The selenium dependent up-regulation of GPx and

247 ThxRed was most marked in BeWo and JEG-3 trophoblastic cell lines but less so in
248 Swan-71 cells.

249

250 Following induction of anti-oxidant expression, the BeWo, JEG-3 and Swan-71 cells
251 were oxidatively stressed with Rotenone and Antimycin in a dose dependent
252 manner. Rotenone and Antimycin block Complex 1 and 3 respectively of the electron
253 transport chain resulting in electron leakage and generation of superoxide ($O^{\bullet-}$) in
254 the mitochondrial matrix. Due to the relatively high concentration and catalytic
255 activity of Mn-SOD, the $O^{\bullet-}$ is rapidly converted to H_2O_2 which is potentially very
256 damaging to cell viability. In these experiments cellular metabolic activity was
257 determined using the Reszaurin end point assay and we investigated whether up-
258 regulation of GPx and ThxRed could protect from mitochondrial oxidative stress.

259

260 As presented above, there was a dose dependent decrease in the cellular activity in
261 BeWo, JEG-3 and Swan-71 when treated with increasing concentrations of
262 Rotenone (100-800nM) and Antimycin (40-320 μ M). Prior incubation with Na Selenite
263 or Selenomethionine was able to protect trophoblast cells from oxidative stress. In all
264 trophoblast cell lines there was considerable protection observed at Rotenone
265 concentrations of 400-200nM. Selenium supplementation of Jeg-3 and BeWo cell
266 lines conveyed protection at Antimycin concentrations 120-320 μ M, with protection
267 against Antimycin concentrations as low as 80 μ M observed in Swan-71 cells. From
268 these observations it would appear that there is a range of stressor concentrations
269 where protection is possible and cells were irreversibly damaged by oxidative stress
270 at higher concentrations. The mechanisms which cause this stress, whether it is lack
271 of respiration, excessive RONS production, apoptosis or necrosis are currently
272 subject to further investigation.

273

274 In a previous study we demonstrated that selenium supplementation could protect
275 trophoblast cells from exogenously applied oxidative stress [8]. We used various
276 forms of peroxides to stress cells and clearly demonstrated the beneficial effects of
277 up-regulation of GPx and ThxRed through Se supplementation. However, the
278 concentrations of peroxides used were probably higher than would be present in
279 trophoblast cells in vivo and the application of exogenous peroxides would
280 presumably have more of an effect in the cytoplasm as the cell absorbs the toxin. In

281 the present experiment we demonstrated a similar protective effect to endogenously
282 generated oxidative stress. Blocking electron transport with Rotenone and Anyimycin
283 is a generally accepted method of generating intra mitochondrial oxidative stress.
284 This is very pertinent to studies on oxidative stress in the placenta during
285 preeclampsia, which is often attributed to mitochondrial, and or endoplasmic
286 reticulum stress. This study clearly shows a link between selenium, the antioxidant
287 proteins GPx and ThxRed and mitochondrial oxidative stress.

288

289 Selenium is an essential micronutrient and without it tissues are susceptible to
290 oxidative stress through loss of function of key enzymes such as GPx and ThxRed
291 [13]. Selenium supplementation also offers an attractive method of maximising anti-
292 oxidant expression in preparation of an oxidative insult [17]. Our previous work in
293 cardiac tissues has shown the benefits of selenium supplementation in patients
294 undergoing coronary by-pass surgery, a clinical scenario where oxidative stress
295 negatively impacts on cardiac recovery [6]. Similarly, selenium supplementation may
296 be of benefit for women who may experience preeclampsia. Indeed our recent work
297 would suggest a link between selenium status and the development of preeclampsia
298 and recent supplementation trials have indicated a possible beneficial effect [14,18].
299 Larger more controlled trials are needed to prove the benefits of selenium
300 supplementation in lowering the incidence or severity of preeclampsia but it is now
301 clear that selenium, through up-regulation of key anti-oxidant proteins can protect
302 against mitochondrial oxidative stress in placental trophoblast cells.

303

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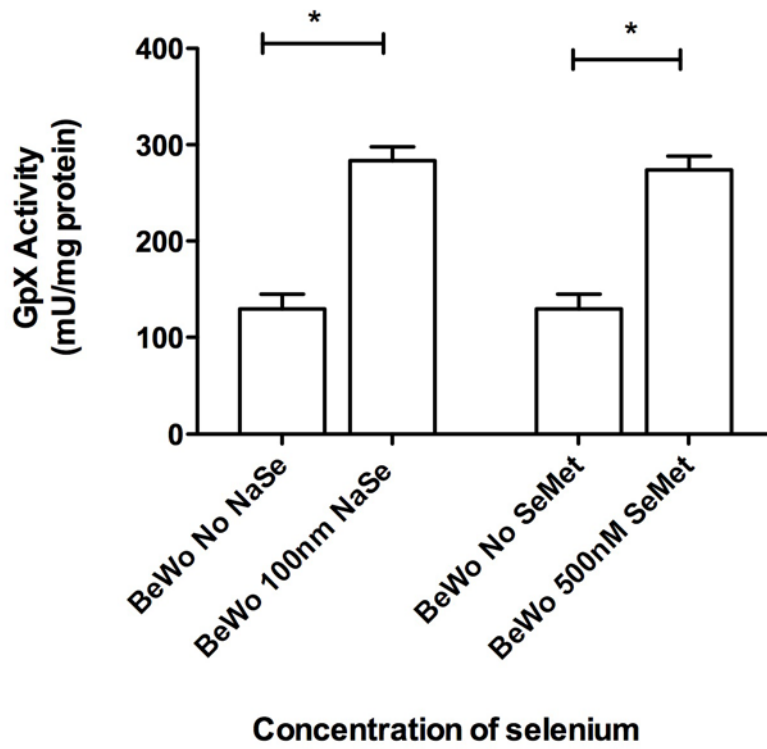
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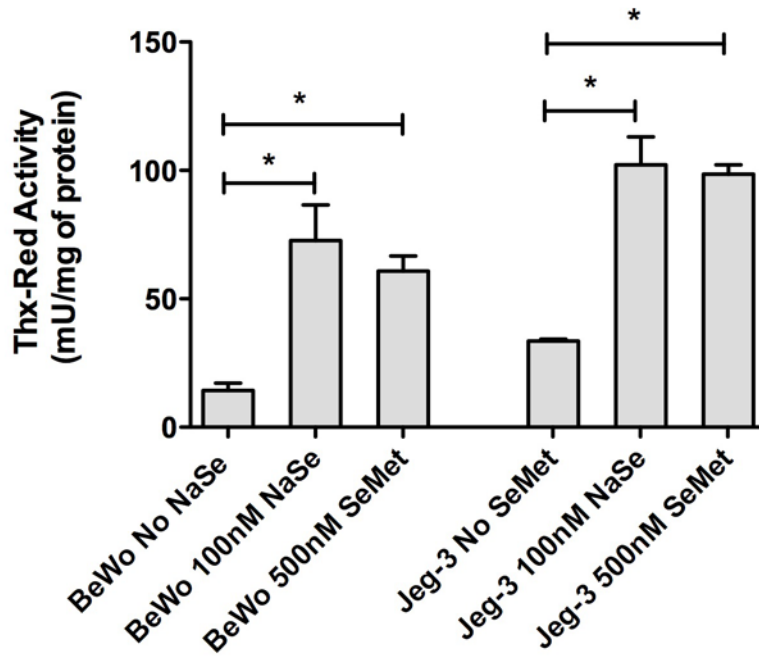
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360 **Figure 1:** Activity of GPx in NaSe and SeMet supplemented BeWo cell lines. Values are
361 presented as mean \pm standard deviation (SD). Significant differences were detected
362 between control and maximum activity in the 100nM NaSe and 500nM SeMet supplemented
363 cell lines (* $p < 0.05$ n=3).

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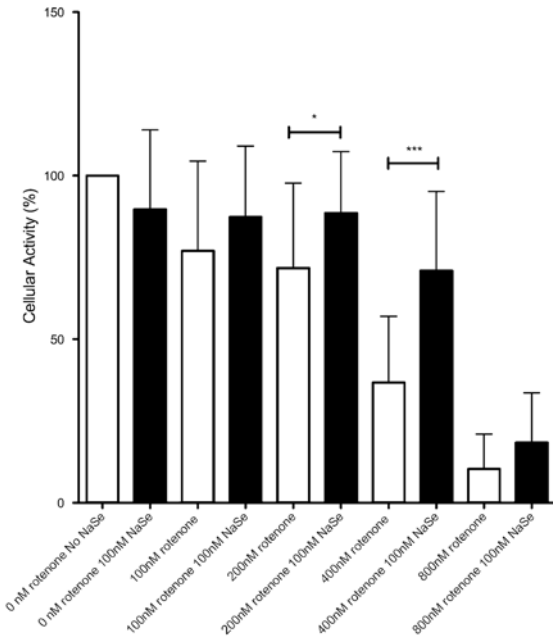
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367 **Figure 2:** Activity of Thx-Red in NaSe and SeMet Supplemented BeWo and JEG-3 cell
368 lines; quantified using Thx-Red inhibition assays. Values are presented as mean \pm standard
369 deviation (SD). Significant differences were detected between control and maximum activity
370 in the 100nM NaSe and 500nM SeMet supplemented cell lines (* $p < 0.05$, $n = 3$).

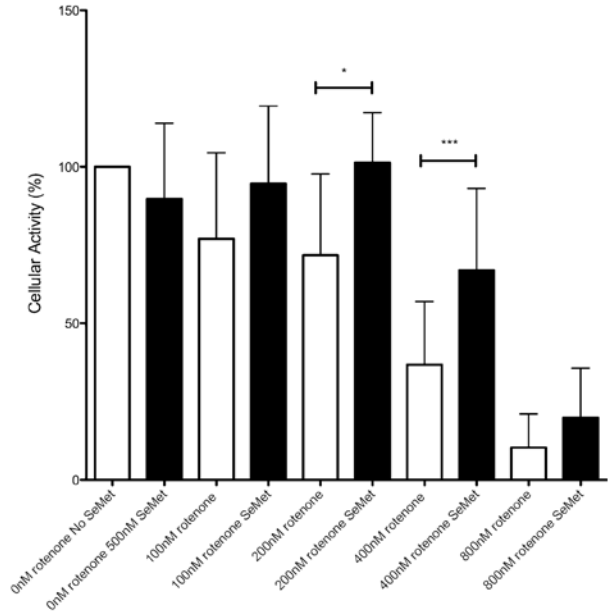
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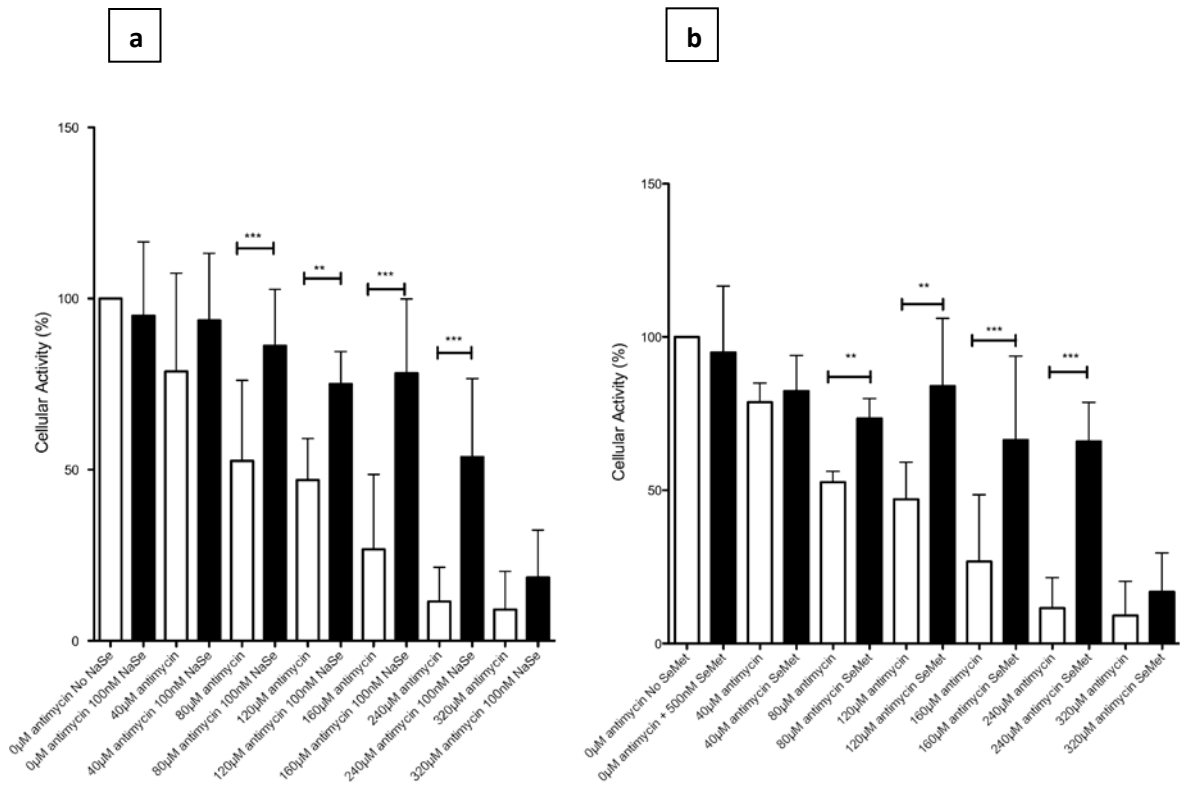


373

374 **Figure 3.** Bewo trophoblast cells treated with NaSe (**a**) or SeMet (**b**) for 24hrs and
 375 subsequently treated with increasing concentrations of rotenone. Values are presented
 376 as mean \pm standard deviation (SD) for each group. Significant differences were detected
 377 between unsupplemented and NaSe and SeMet supplemented cell lines when treated with
 378 200nM and 400nM concentrations of rotenone (* $p < 0.05$, *** $p < 0.001$, $n = 9$).

379

380



381

382 **Figure 4:** Swan-71 trophoblast cells treated with NaSe (A) or SeMet (B) for 24hrs
 383 and subsequently treated with increasing concentrations of antimycin. Values are
 384 presented as mean ± standard deviation (SD) for each group. Significant differences were
 385 detected between unsupplemented and NaSe and SeMet supplemented cell lines when
 386 treated with 80-240µM concentrations of antimycin (** $p < 0.01$, *** $p < 0.001$ n=9).

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