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Detoxification of the Model Sensitiser 2,4-Dinitrochlorobenzene in Reconstructed Human Epidermis: Repeated Exposure Scenario.

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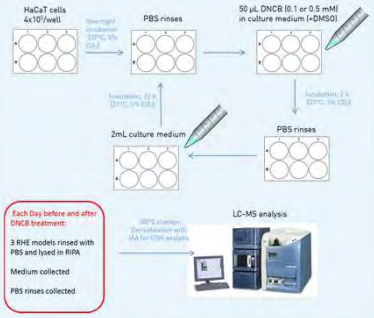
Introduction

We used 2,4-dinitrochlorobenzene (DNCB) to investigate the effects of repeated chemical exposure on Glutathione (GSH) lifecycle in reconstructed human epidermis (RHE). We demonstrated that the RHE model could be used over a three-day period. The amount of conjugate (DNP-SG) increased between the first and second exposure, while GSH repletion to basal level was maintained. These data suggest that skin defence mechanisms might be activated in response to exposure to model sensitisers, resulting in their accelerated clearance.

Example of RHE model in inserts. *The 0.5 cm^2 models were purchased after 17 days of culture, shipped to our laboratory within two days, placed into 2mL of fresh culture medium upon receipt and left to equilibrate overnight in an incubator (37 °C, 5 % CO_2). Experiments were carried out over a 3-day period. The medium was refreshed every 24 hours.*

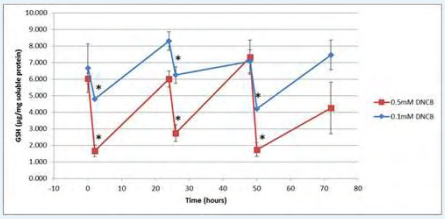
Methods

- Cytotoxicity: MTT assay, 50µL of DNCB solution (range 0.05-10mM) for 24 hours followed by 3 hours MTT assay
- Multiple exposure to DNCB: see experimental scheme on the right
- Lysing procedure: RHE lysed in 2mL RIPA buffer (Soniprep150plus, amplitude 6.5, 5 times 1 minute burst)
- LC-MSMS: GSH (IAA derivatized, m/z 366.02 >173.80) + $^{13}C_2$,^{15}N-GSH (GSH m/z +3) , GSSG (m/z 613.10 > 355.10) and DNP-SG (m/z 474.1 > 242.1) were quantified
- Western blot: Determination of Nrf2 activation, quantification against actin, positive control NQO1 activation

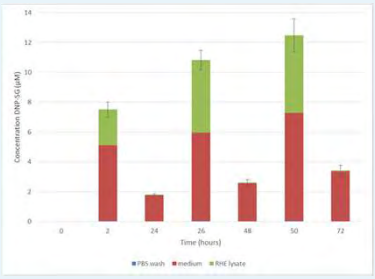


Daily treatment with DNCB. *The RHE were treated for 2 hours daily, rinsed with PBS and placed into fresh medium for 22 hours (recovery period). Models were lysed before and after each treatment (n = 3)*

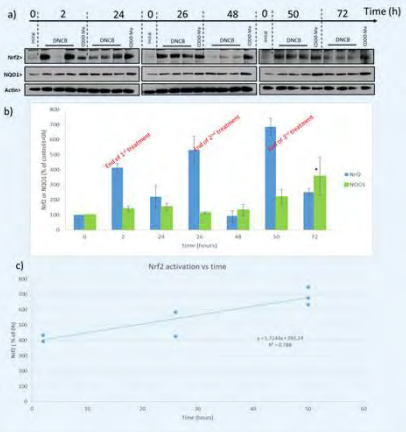
Results



72 hour GSH cycle in RHE samples treated daily with DNCB. *The RHE models were treated with 50µL of DNCB solutions prepared in RHE growth medium containing 1% DMSO (n=3). After each recovery period, GSH levels are replenished to basal level (*) Statistical difference between GSH level in treated RHE samples and RHE samples lysed before treatment (t= 0, 24 and 48 hours) Student's t-test, p value <0.05.*



DNP-SG conjugate formation and excretion in the RHE model. *The DNCB doses applied at t=0, 24 and 48 hours was 0.5mM. After each DNCB treatment (t=2, 26 and 50 hours), the total amount of DNP-SG in the RHE samples increases. The amount of DNP-SG in the medium at the end of the 22 hour recovery period also increases at each exposure. Cumulative data presented for each time point includes PBS washes, RHE growth medium and RHE lysates (n=3).*



Daily DNCB dosing releases Nrf2 exclusively during exposure time. *a) Typical Western Blot showing Nrf2 release during exposure to DNCB (n=3). Data normalised to actin, NQO1 enzyme measured as positive control of Nrf2 activation, positive control CDDO-Me (n=1). For t=0h three control samples were pooled to generate a single t=0 sample , with value fixed at ratio 1 (100% baseline value) and used to calculate the "ratio over controls" for the other samples. b) Data illustration for Nrf2 and NQO1 as a function of time. DNCB induced an increase in Nrf2 level of 4-7 fold at t= 2, 26 and 50h during exposure and decreases back to 1-3 fold, NQO1 activation statistically significant at t=72h (student t-test, p<0.1) c) The release of Nrf2 was increased after each exposure to DNCB. The mean Nrf2 values of time points 2h, 26h and 50h were statistically different (ANOVA, p<0.05).*

The GSH level in RHE skin models is stable at homeostasis (5-9 µg/mg soluble protein). Applying a low dose of 0.1-0.5 mM DNCB (equivalent to 2-10 µg/cm²) partially depletes the GSH stock within 2 hours, which is then replenished to basal level during the recovery period (figure on the left).

Concomitantly, the activation of the Nrf2 pathway can be observed in the RHE model after each exposure) with an up-regulation of Nrf2 level in the cytosol between each regimen (figure on the right). The induction of Nrf2 was correlated to an increase in available GSH (upregulation of the enzymes GCL to increase GSH synthesis), which was indirectly measured by monitoring the formation of the dinitrophenyl glutathione conjugate in both medium and RHE lysate (figure in the middle).

When three consecutives treatments with 0.5mM DNCB were used, the overall level of dinitrophenyl glutathione (DNP-SG) formed (model lysate and medium) increased between the first and second exposure and stayed high for the third exposure. This demonstrated the potential of RHE models to be used for the induction of Phase II metabolism enzymes such as GSTs.

Conclusion and perspectives

- Repeat low dosing of electrophiles promotes up-regulation of the defence mechanisms linked to the GSH pathway in skin to protect against exogenous compounds.
- 3D skin models such as the RHE appear to be a suitable substitute to *ex vivo* human skin to study metabolism in a repeated exposure scenario (low dose but successive treatments).
- Skin metabolism as a defence system include other pathways that should be investigated (Phase II enzymes: glucuronidation, sulphation, methylation). Conversely, examples of activation of pro-haptens (Phase I enzymes) might also be demonstrated in these 3D in vitro models.

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References: Spriggs et al., Toxicological Sciences,2016 Nov;154(1):5-15.