Evaluating the effects of nacre on human skin and scar cells in culture

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Supporting Information

S1. Experimental

S1.1 Materials

Pinctada margaritifera shells were provided by Pearl Technologies Pty Ltd, which were grown in the waters of the Abrolhos Islands, Western Australia. The decalcified organic conchiolin layer was removed by wet sand blasting of the shell followed by gentle brushing to remove any dust particles that might otherwise contaminate the samples. Inner nacreous layer was then scraped using a surgical scalpel and stored at room temperature for a maximum of 2 weeks.

S1.2 Scanning Electron Microscopy

Scraped nacre above from the inner layer of the shell was mounted on SEM stubs (ProSciTech, Cat.# G040). Samples were coated with 4 nm of platinum. Images were taken using scanning electron microscope (Zeiss 1555, VP-FESEM) at 4-5 kV at 30µm aperture. Images were analyzed with the image analysis software ImageJ (NIH).[1]

S1.3 Cell culture

A human derived immortalized keratinocyte cell line, HaCaT [2] and two human primary dermal (fibroblast) cell cultures from normal skin and normal scar were used. All three cell types were cultured in Dulbecco's Modified Eagle's Medium (DMEM/F12 - GlutaMAX; Invitrogen Gibco) supplemented with 10% fetal bovine serum (FBS; Invitrogen Gibco) and 1% penicillin/streptomycin (Invitrogen Gibco). The cells were incubated at 37°C in an atmosphere of 5% CO_2. Primary cells used were between the passages 7-10. Nacre was sterilized using UV sterilization technique in the tissue culture hood for 15 min prior its dissolution in media. Fresh nacre solution was prepared before every experiment.

S1.4 Cell Viability

Cell viability was determined using a LIVE/ DEAD Viability/Cytotoxicity Kit (Invitrogen, UK) which measures the membrane integrity of cells,[3, 4] as per manufacturer's protocol. In brief, 20000 cells were seeded in each well in a 24 well plate and treated with scraped nacre at various concentrations in cell culture media (DMEM F-12 containing 10% FBS and 1% Penicillin/ Streptomycin) and incubated for 24 h or 72 h in the humidified incubator at 37°C with 5% CO_2. At the stipulated time (24h and 72h), cells were washed with PBS (3 times) and then stained with calcein (100 µL, 1µM)/ ethidium bromide (100 µL, 2 µM) in PBS and incubated in the humidified incubator at 37°C and 5% CO_2 for 30 min. Images were captured using an Olympus IX71 inverted microscope with a 20 x objective with fixed exposure time. Both live and dead cells were counted using Image J with cell counter plug in. Experiments were performed in triplicate. Minimum of fifty images were captured per condition.

S1.5 Reactive Oxygen Species (ROS)

ROS was measured using the ROS assay kit (Oxiselect ROS assay kit, Cat.# STA 342, Cell Biolabs) following manufacturer's protocol. In brief, 6000 cells were seeded in a 96 well plate and incubated in the humidified incubator at 37°C with 5% CO_2 for 24h. Next day, wells were washed with PBS (3 times) and incubated with 2', 7'-dichlorodihydrofluorescin diacetate (DCFH-DA) solution (0.1 x, 100 µL/ per well) for 1 h in the humidified incubator at 37°C with 5% CO_2. DCFH-DA solution was removed and the wells washed with 3 x PBS. Cells were then treated with scraped nacre solution in culture media at a specified concentration for 24 h, wells were washed with 3 x PBS and cells were lysed using the lysis buffer provided (1 x, 100µL/ per well, incubated for 20 min. at room temperature) before reading the plate at 480 nm excitation/ 530 nm emissions using the plate reader. Experiments were performed in triplicate.

S1.6 Cell Body Area

Cell size was measured using Image J software (NIH).[1] A minimum of 25 cells were randomly selected from the fluorescence images and their area was measured. Values reported as mean ± standard error mean.

S1.7 Statistics

The results for cell viability, ROS experiments and cell area are expressed as mean ± standard error mean (SEM) and analysed by analysis of variance (ANOVA). Significance was evaluated using Bonferroni and Turkey's post-hoc analysis and set at 95% confidence ($p < 0.05$).

Supporting Figures

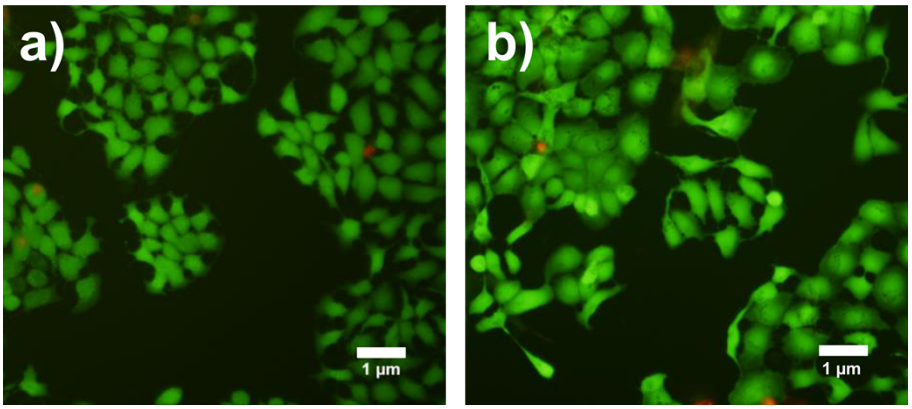

Figure S1: Cell morphology post calcein AM/ ethidium bromide staining and imaged using fluorescent microscopy. HaCaT cells were treated with various concentrations of nacre for 24h, stained and imaged. a) Untreated (control), b) HaCaT cells treated with 2.5 mg/mL nacre. Scale bar 1µm.

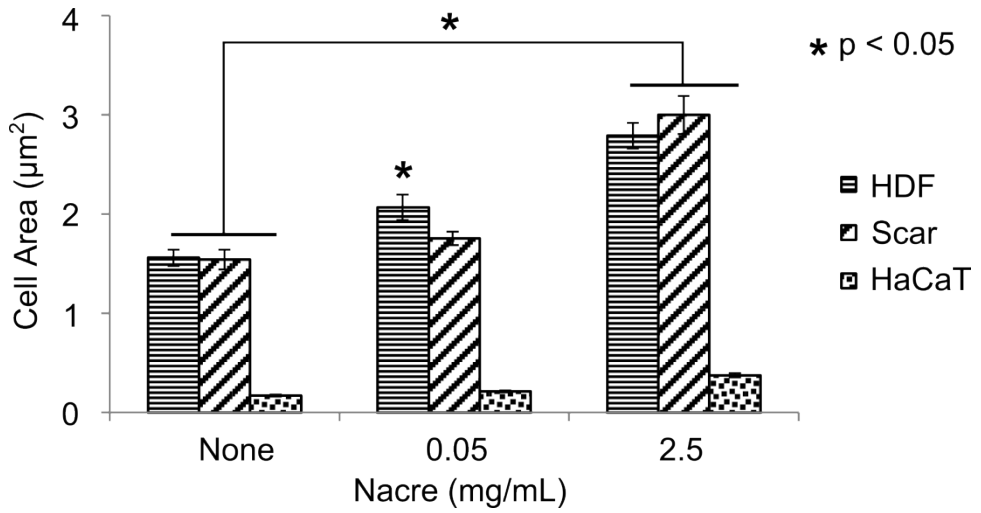

Figure S2: Cell area size showing increase in the cell area post incubation with nacre. Cell area was measured from the fluorescent images of live cells taken for viability assay. 'None' is the untreated control. Data presented as average ± SEM (n>25). Significance was set at * $p < 0.05$ using bonferroni post hoc test in one way ANNOVA

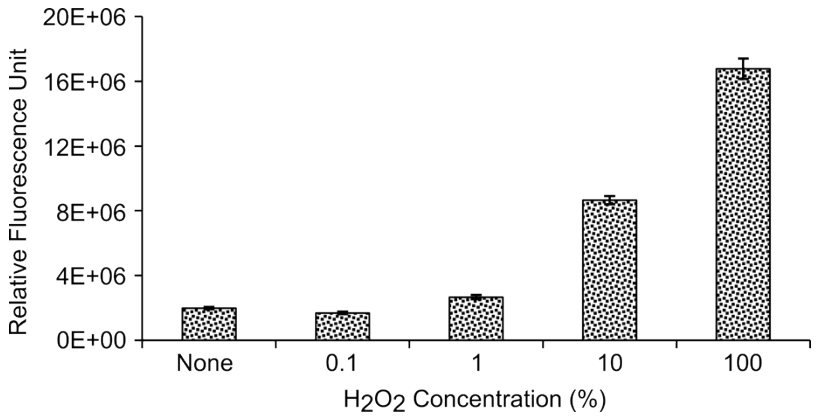

Figure S3: Reactive oxygen species (ROS) assay showing increase in ROS levels in cells stressed with various concentrations of H_2O_2 in dose dependent manner. Human dermal skin fibroblasts cells were incubated with various concentrations of H_2O_2 for the specified period of time to generate the standard curve. Cells were then incubated with 2', 7'- dichlorodihydrofluorescin diacetate (DCFH-DA) solution which fluoresce in the presence of reactive oxygen species. 'None' is the untreated control. Data presented as average ± SEM (n=3).

References

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