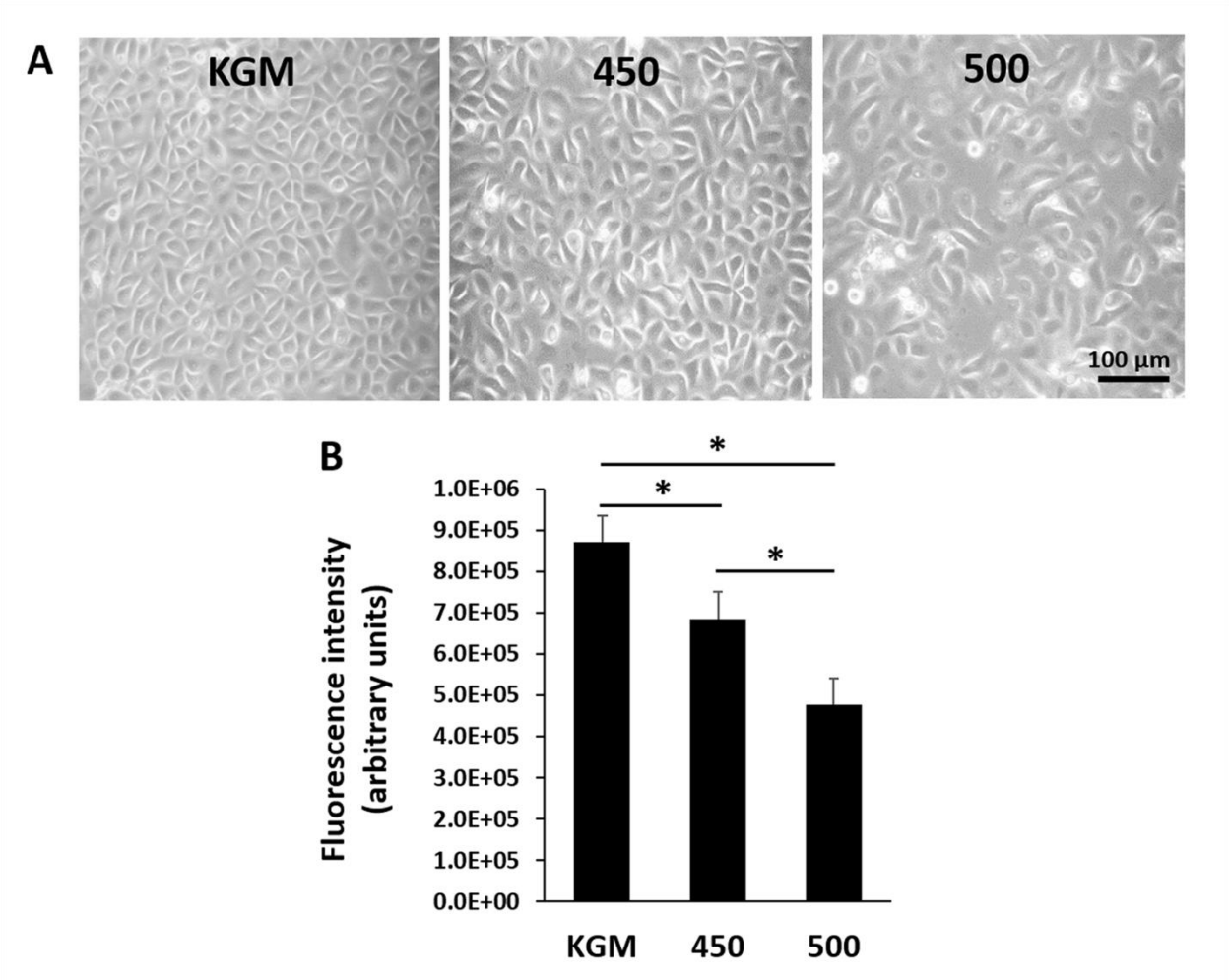
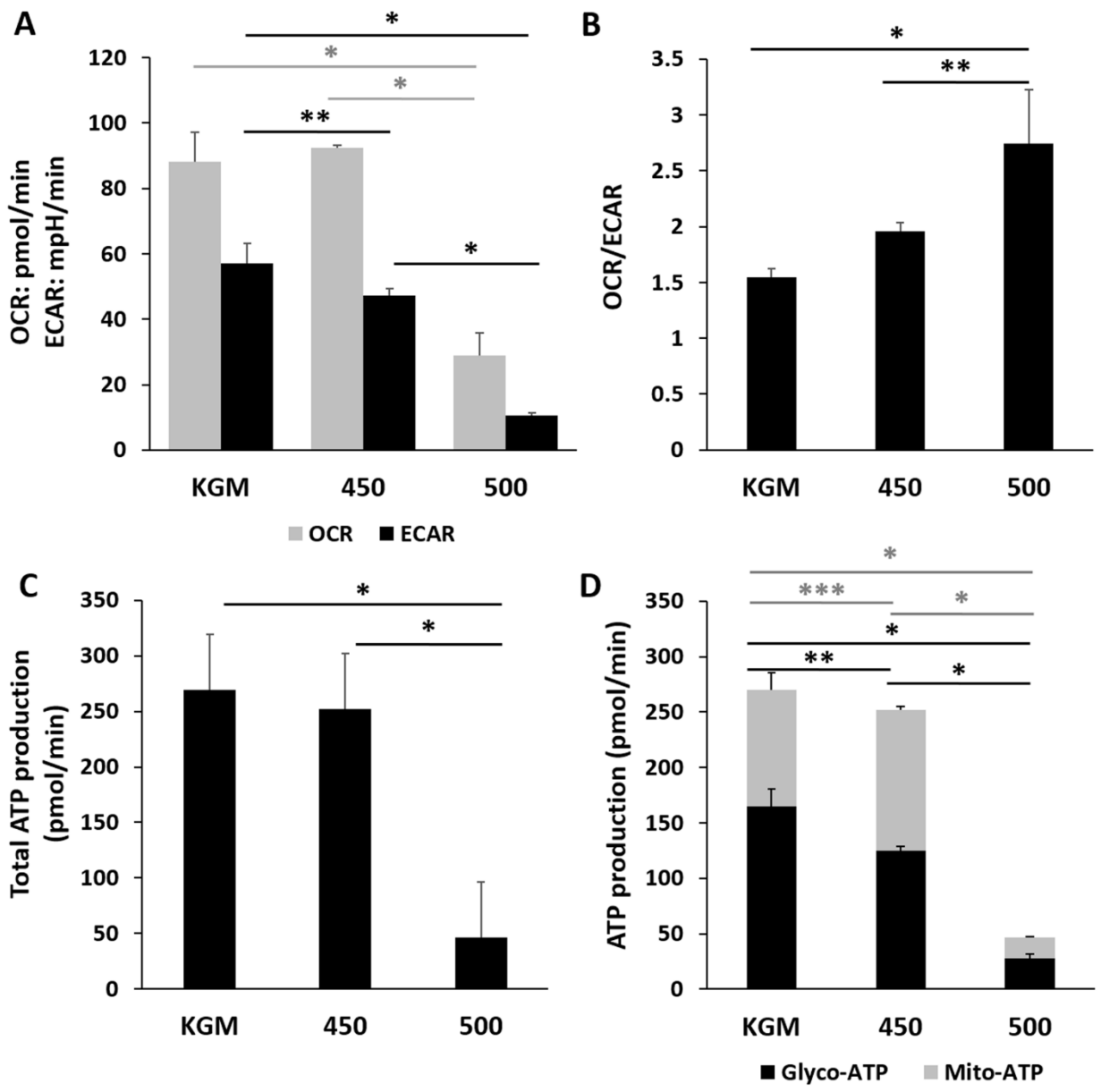
Supplementary Material

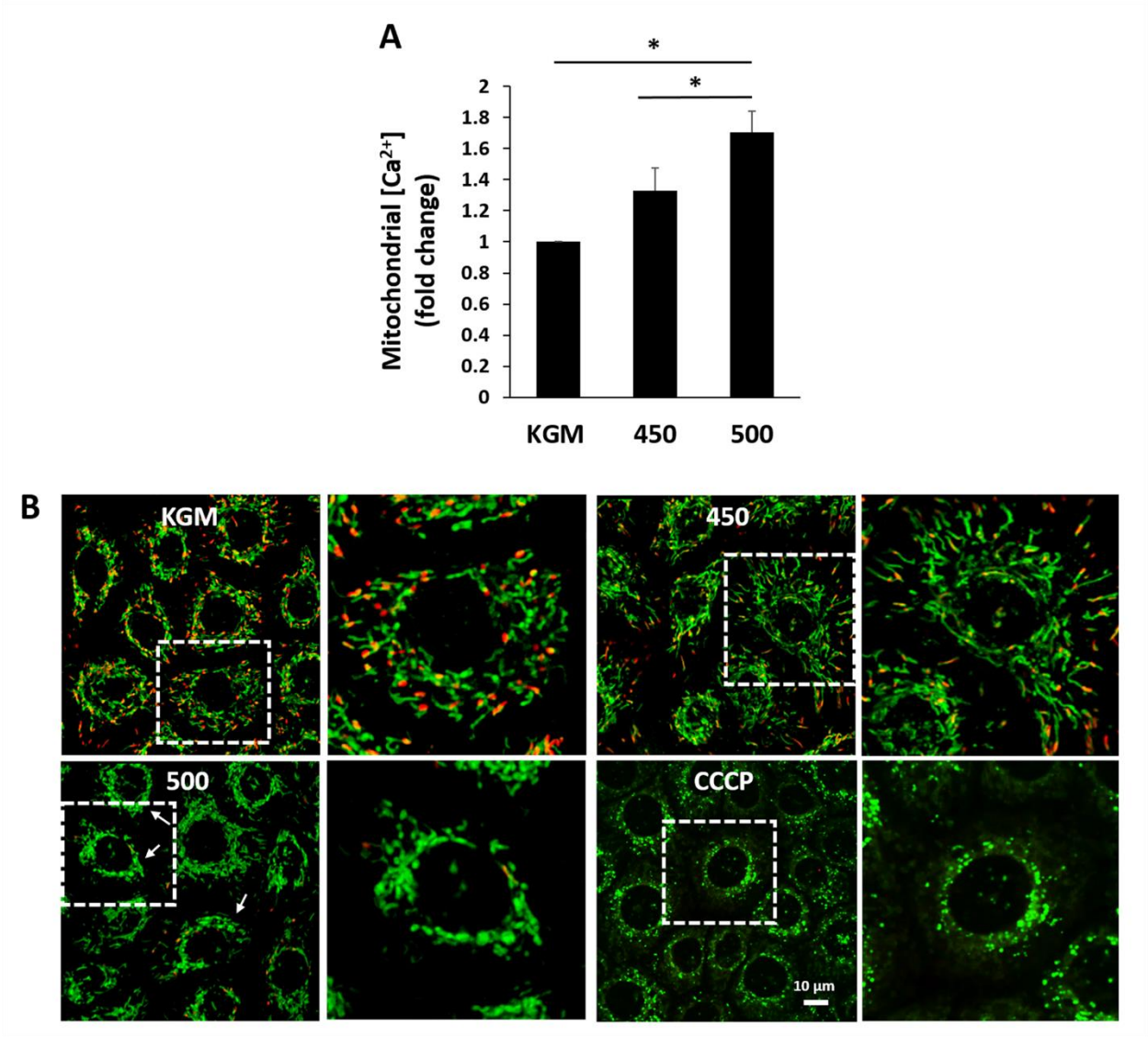
Supplementary Figure 1: Corneal epithelial cells exhibit reduced growth in hyperosmolar culture that is not due to an increase in IGFBP-3. hTCEpi cells were cultured in growth media with increasing levels of salt for 24 hours. (A) Phase contrast microscopy of hTCEpi cells cultured under increasing levels of hyperosmolar stress. Scale bar: 100 μm. (B) Cell proliferation was measured using a CyQuant assay. There was a decrease in cell proliferation that corresponded to increasing concentrations of salt (*P<0.001, One-way ANOVA, SNK multiple comparison test, n=3).



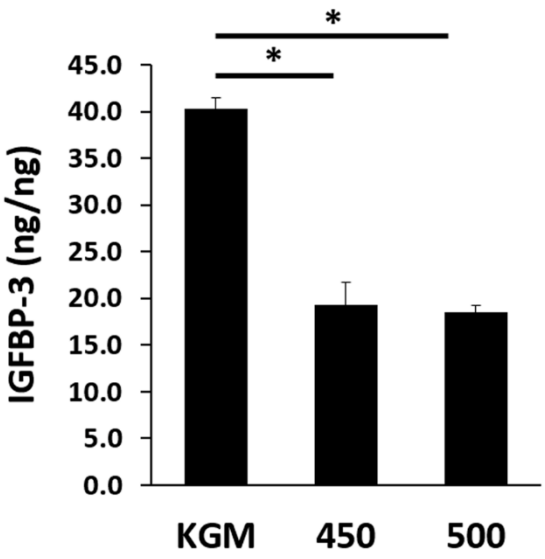
Supplementary Figure 2: Hyperosmolar culture differentially affects glycolysis in corneal epithelial cells, shifting cells toward a respiratory phenotype. hTCEpi cells were cultured in growth media with increasing levels of salt for 24 hours and bioenergetics were measured using a Seahorse XFp real-time ATP rate assay. (A) Glycolysis was decreased at 450 mOsM and further decreased at 500 mOsM. Mitochondrial respiration was also decreased at 500 mOsM, but was unaffected at the lower osmolarity (*P<0.001, **P=0.018, One-way ANOVA, SNK multiple comparison test, n=3). (B) The OCR/ECAR ratio shifted towards a respiratory phenotype during hyperosmolar culture (*P=0.021, **P=0.048, One-way ANOVA, SNK multiple comparison test, n=3). (C) Total ATP production was unaffected when cultured in 450 mOsM, but was significantly decreased at 500 mOsM (*P<0.001, One-way ANOVA, SNK multiple comparison test, n=3). (D) ATP generated from glycolysis was decreased at 450 mOsM and further decreased at 500 mOsM (*P<0.001, **P=0.005). In contrast to this, ATP produced by mitochondrial respiration was increased during culture in 450 mOsM, but was reduced by culture in 500 mOsM (*P<0.001, ***P=032, One-way ANOVA, SNK multiple comparison test, n=3). All data expressed as mean ± standard deviation. Graphs representative of a single experiment repeated three times. KGM: isotonic keratinocyte growth media; 450: 450 mOsM; 500: 500 mOsM; OCR: oxygen consumption rate; ECAR: extracellular acidification rate.


A
OCR: pmol/min
ECAR: mpH/min
120
100
80
60
40
20
0
KGM
450
500
OCR
ECAR
B
OCR/ECAR
3.5
3
2.5
2
1.5
1
0.5
0
C
Total ATP production (pmol/min)
350
300
250
200
150
100
50
0
D
ATP production (pmol/min)
Glyco-ATP
Mito-ATP

Supplementary Figure 3: Mitochondrial calcium concentration increases in corneal epithelial cells in hyperosmolar culture. hTCEpi cells were cultured in growth media with increasing levels of salt for 24 hours. (A) There was an increase in mitochondrial calcium associated with hyperosmolarity that peaked at 500 mOsM (*P<0.001, One-way ANOVA, SNK multiple comparison test).(B) JC-1 staining showed a reduction in mitochondrial polarization in 450 mOsM culture. JC-1 monomers representing depolarized mitochondria shown in green and JC-1 aggregates representing polarized mitochondria shown in red. At 500 mOsM, mitochondria were almost completely depolarized and areas of fragmentation were visible (arrows). CCCP, an uncoupler of oxidative phosphorylation, was used as a positive control. White dashed square indicates are of zoom in adjacent image. Scale bar: 10 μm. KBM: keratinocyte basal media. Number indicates mOsM. Images representative of 3 repeated experiments.


A
*
*
Mitochondrial [Ca2+] (fold change)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
KGM
450
500
B
KGM
450
500
CCCP
10 μm

Supplementary Figure 4: Hyperosmolar stress downregulates IGFBP-3 in growth media. IGFBP-3 levels were reduced when cultured in hyperosmolar growth media (*P<0.001, One-way ANOVA, Holm-Sidak multiple comparison test, n=3). IGFBP-3 was normalized to total cell lysate protein. Data represented as mean ± standard deviation. Graphs representative of a single experiment repeated three times. KGM: isotonic keratinocyte growth media; 450: 450 mOsM; 500: 500 mOsM.



Supplementary Figure 5: Reduced cell number in hyperosmolar culture was not due to cytotoxicity. hTCEpi cells were cultured in basal media under isotonic or hyperosmolar conditions (450 mOsmol) for 24 hours, with or without 500 ng/ml rhIGFBP-3. Cytotoxicity was measured using an LDH assay. All cells exposed to hyperosmolar stress showed a small but significant increase in cytoxicity (*P<0.001, One-way ANOVA, SNK multiple comparison test, n=3). No difference in cytotoxicity was observed in cells treated with rhIGFBP-3. All data expressed as mean ± standard deviation. Graphs representative of a single experiment repeated three times. KBM: isotonic keratinocyte basal media; 450: 450 mOsM; BP3: IGFBP-3.