Investigation of the Synergistic Effect of PHI Components on MMP Expression on Fibroblasts Cells

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All Studies were analyzed by ANOVA followed by Tukey's and Dunnett's post-hoc tests, when statistical significance was detected. In most cases, the results of both tests were in agreement. Dunnnett's test was a bit less strict, the results of which are denoted by the asterix marks (*p<0.05, pp<0.01, pp<0.001). All comparisons were made against the control, which contained no additional PHI ions.

Use of HFF-1 Cells

The skin is composed of a deep dermal fibroblast matrix beneath an epidermal layer of stratified keratinocytes. Upon injury, the epidermal layer is damaged, exposing the underlying fibroblasts. During wound care, these fibroblasts are exposed to the host of ointments and dressings currently on the market. As the dermal fibroblasts represent the majority of cells that may be present during the care of dermal wounds, we chose Human Foreskin Fibroblasts (HFF-1, ATCC Cat No. SCRC-1040) as a model system for *in vitro* analysis of the various ions. Per ATCC, this is a normal skin fibroblast cell line that was acquired from two newborn males.

STUDY #1. PHI B12007 on Human Foreskin Fibroblasts

Cells were treated with PHI-B12007. According to our records, this consisted of PHI consists of 10mg/ml KCl, 75ug/ml RbCl, 2.5ug/ml $CaCl_2$, 1.2ug/ml $ZnCl_2$. This composition represented 100% PHI in this study.

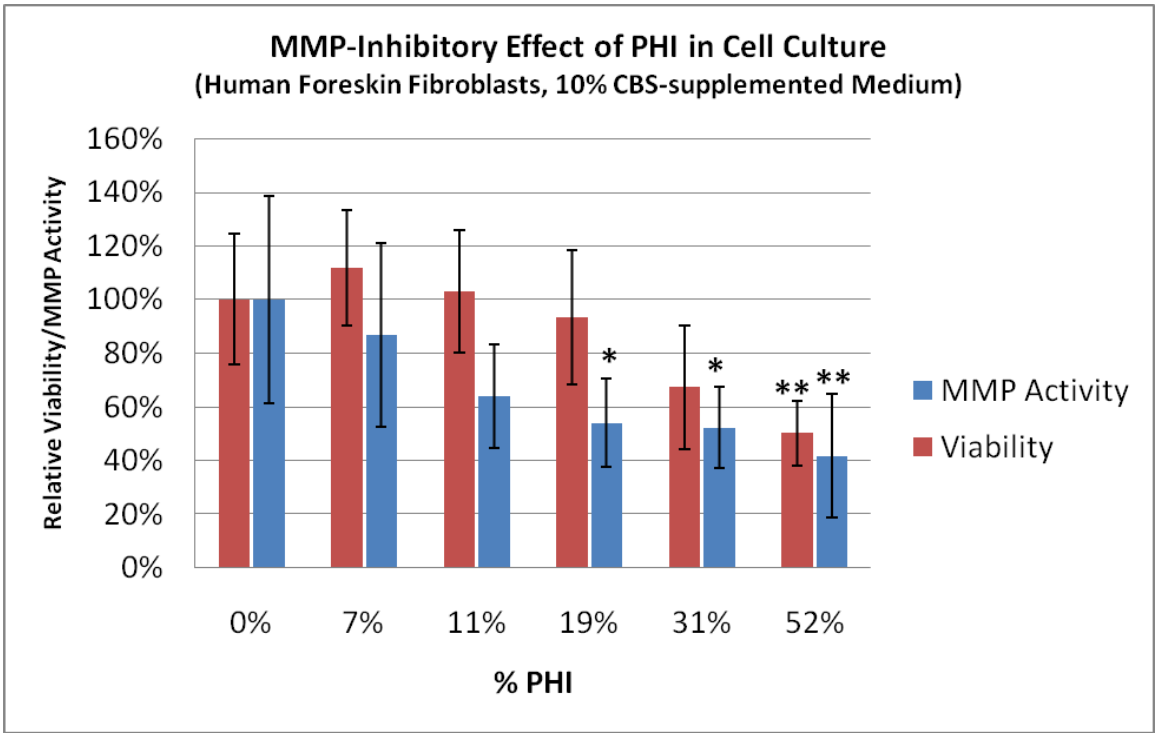

Figure 1. PHI B12007 was found to inhibit MMP Expression in cultures supplemented with 10% calf bovine serum. This was observed in a dose-dependent manner. Approximately 45% reduction of MMP activity was observed with insignificant effect on cell viability.

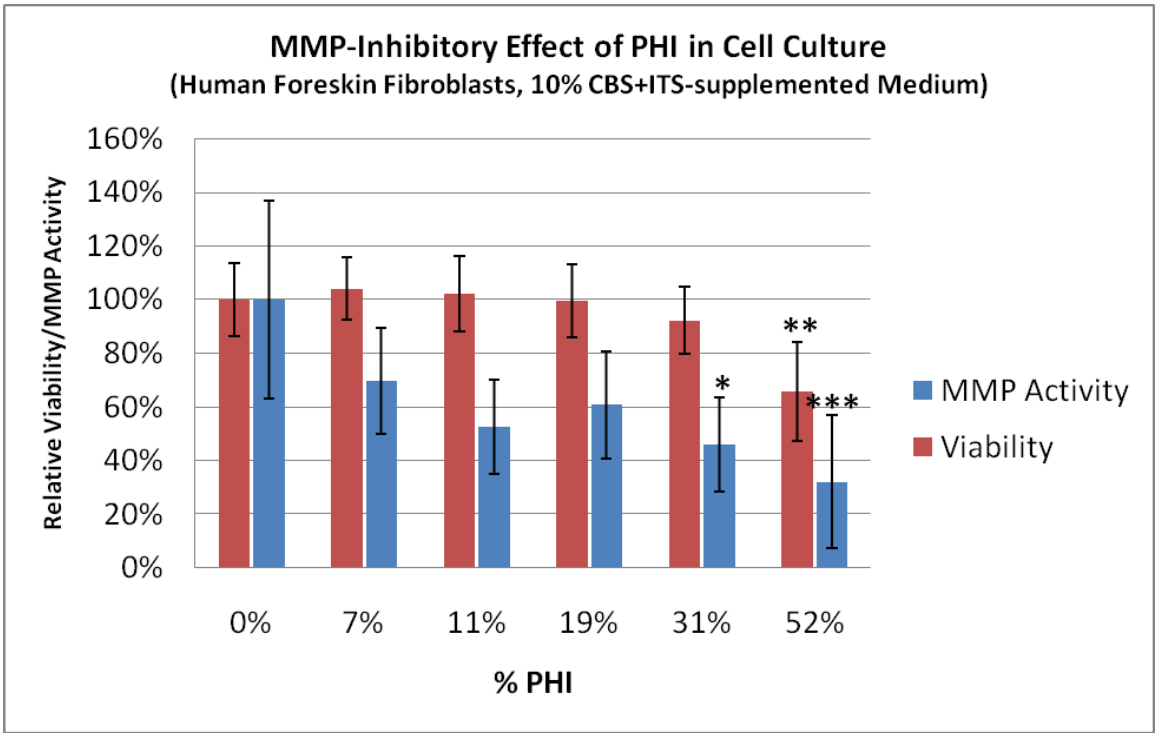

Figure 2. PHI B12007 was found to inhibit MMP Expression in cultures treated with 10% calf bovine serum and ITS (Insulin-Transferrin-Selenium) supplement. This was observed in a dose-dependent manner. Approximately 45% reduction of MMP activity was observed with insignificant effect on cell viability.

STUDY #2: Modified PHI and Full Factorial Analysis on Optimal PHI Compositions

A 3^4 full factorial design was performed to determine the optimal PHI component ration in cells culture. The chosen factors and levels are described below. **It should be noted here that the PHI composition in the previous study was suspected of being incorrectly reported to us, per Greystone PHI records/reports binder. My suspicions arose from the fact that KCl was reported at 3 ORDERS OF MAGNITUDE HIGHER CONCENTRATION than all the other components. For this reason, I used a value of 10ug/ml instead of 10mg/ml as the 1X PHI B12007 composition for this study**.

Table 1. 3^4 Factorial Analysis of PHI Components on HFF Cells in culture.

Factors:	Zn	Rb	K	Ca	
Levels:	**Zn**	0	1.2	12	ug/ml
	Rb	0	7.5	75	ug/ml
	K	0	10	100	ug/ml
	Ca	0	2.5	25	ug/ml

All treatments were replicated a minimum of 5 times, some (as in the case of the controls), were replicated up to 20 times. Thus, the majority of samples had n=5, while others had n=10, 15m or 20. A total of 480 samples (81 combinations) were tested. Cells were seeded at 25,000 cells/well, grown 36hrs prior to treatment. Treatment was commenced by aspirating the media, adding 400ul fresh media, and then adding 100ul 5X treatments. Treatments were prepared in 4-24-well plates and applied with the aid of a multichannel pipette. Cells were then returned to the incubator and allowed to grow ~72hrs. At that time, media were collected (200ul). The remainder media was then aspirated and cells were treated with 200ul MTS/media for 1hr to test proliferation/cytotoxicity. The collected media was analyzed by the TNO211 peptide cleavage assay for MMP activities. The complete cell culture medium used contained phenol-free DMEM + 10% CBS + 0.15% Na Bicarbonate + Na Pyruvate + MEM NEAA + Ab/Am.

Table 2. Treatment Conditions Tested in Cell Culture. All concentrations are in ug/ml for each ion.

Treatment	Zn	Rb	K	Ca	n
1	**0**	**0**	**0**	**0**	**20**
2	0	0	0	2.5	10
3	0	0	0	25	10
4	0	0	10	0	10
5	0	0	10	2.5	5
6	0	0	10	25	5
7	0	0	100	0	10
8	0	0	100	2.5	5
9	0	0	100	25	5
10	0	7.5	0	0	10
11	0	7.5	0	2.5	5
12	0	7.5	0	25	5
13	0	7.5	10	0	5
14	0	7.5	10	2.5	5
15	0	7.5	10	25	5
16	0	7.5	100	0	5
17	0	7.5	100	2.5	5
18	0	7.5	100	25	5
19	0	75	0	0	10
20	0	75	0	2.5	5
21	0	75	0	25	5
22	0	75	10	0	5
23	0	75	10	2.5	5

Treatment	Zn	Rb	K	Ca	n
1	**0**	**0**	**0**	**0**	**20**
42	1.2	7.5	10	25	5
43	1.2	7.5	100	0	5
44	1.2	7.5	100	2.5	5
45	1.2	7.5	100	25	5
46	1.2	75	0	0	5
47	1.2	75	0	2.5	5
48	1.2	75	0	25	5
49	1.2	75	10	0	5
50	1.2	75	10	2.5	5
51	1.2	75	10	25	5
52	1.2	75	100	0	5
53	1.2	75	100	2.5	5
54	1.2	75	100	25	5
55	12	0	0	0	10
56	12	0	0	2.5	5
57	12	0	0	25	5
58	12	0	10	0	5
59	12	0	10	2.5	5
60	12	0	10	25	5
61	12	0	100	0	5
62	12	0	100	2.5	5
63	12	0	100	25	5

Treatment	Zn	Rb	K	Ca	n
24	0	75	10	25	5
25	0	75	100	0	5
26	0	75	100	2.5	5
27	0	75	100	25	5
28	1.2	0	0	0	10
29	1.2	0	0	2.5	5
30	1.2	0	0	25	5
31	1.2	0	10	0	5
32	1.2	0	10	2.5	5
33	1.2	0	10	25	5
34	1.2	0	100	0	5
35	1.2	0	100	2.5	5
36	1.2	0	100	25	5
37	1.2	7.5	0	0	5
38	1.2	7.5	0	2.5	5
39	1.2	7.5	0	25	5
40	1.2	7.5	10	0	5
41	1.2	7.5	10	2.5	15

Treatment	Zn	Rb	K	Ca	n
64	12	7.5	0	0	5
65	12	7.5	0	2.5	5
66	12	7.5	0	25	5
67	12	7.5	10	0	5
68	12	7.5	10	2.5	5
69	12	7.5	10	25	5
70	12	7.5	100	0	5
71	12	7.5	100	2.5	5
72	12	7.5	100	25	5
73	12	75	0	0	5
74	12	75	0	2.5	5
75	12	75	0	25	5
76	12	75	10	0	5
77	12	75	10	2.5	5
78	12	75	10	25	5
79	12	75	100	0	5
80	12	75	100	2.5	5
81	12	75	100	25	15

Optimal combinations were detected upon performing ANOVA followed by the post-hoc analysis on the raw MMP concentrations detected in the conditioned medias. Furthermore, a global control (the average of all control samples) was calculated and relative MMP expression levels were calculated and analyzed. **The results implicate ONLY 2 SPECIFIC COMBINATIONS that effectively reduced MMP levels**. These are indicated in the following charts. MTS analysis revealed highly viable cells at each of these treatment conditions.

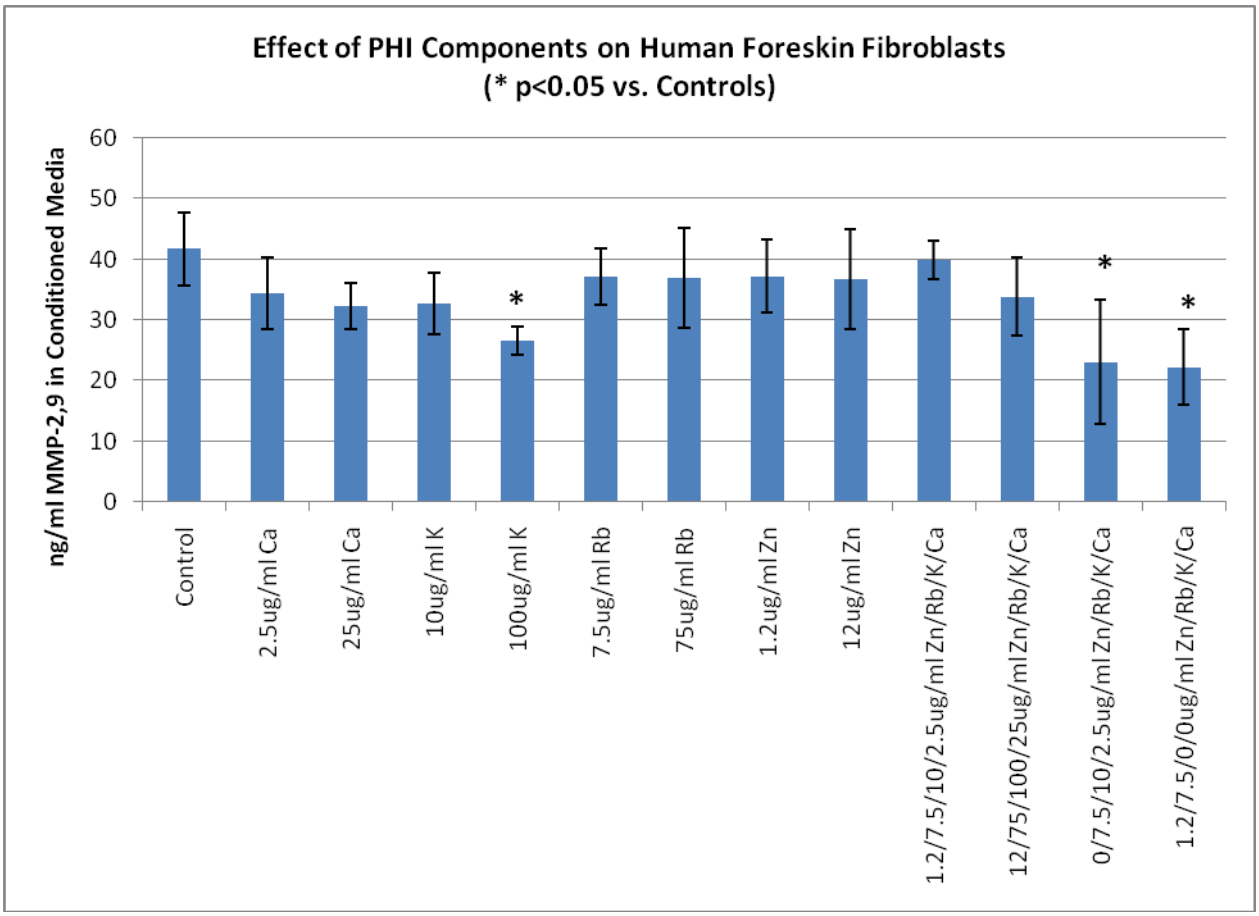

Figure 3. Comparisons Between Selected Treatment Conditions. The total concentrations of MMP detected in the supernatant medias of cell treated with the respective treatment conditions of the PHI components. All 81 conditions in Table 1 were analyzed by ANOVA followed by Tukey's multiple comparison tests. Only 0/7.5/10/2.5ug/ml and 1.2/7.5/0/0ug/ml Zn/Rb/K/Ca were found to result in a statistically significant reduction of MMP activity in the supernatant medias following ~72hr treatment.

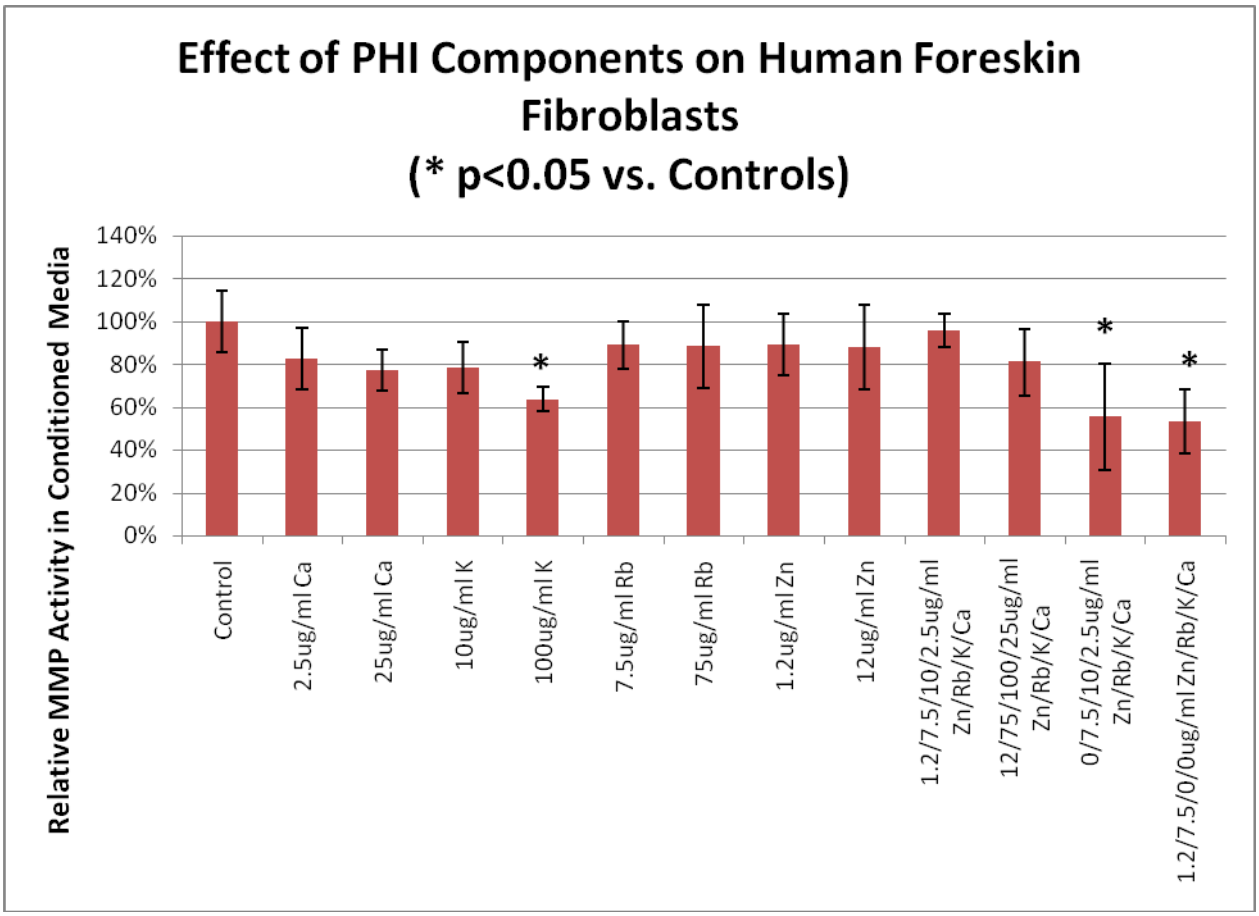

Figure 4. Comparisons Between Selected Treatment Conditions. Relative MMP concentrations vs. non-treated controls are presented for several key treatment conditions of the PHI components. All 81 conditions in Table 1 were analyzed by ANOVA followed by Tukey's multiple comparison tests. Only 0/7.5/10/2.5ug/ml and 1.2/7.5/0/0ug/ml Zn/Rb/K/Ca were found to result in a statistically significant reduction of MMP activity in the supernatant medias following ~72hr treatment.

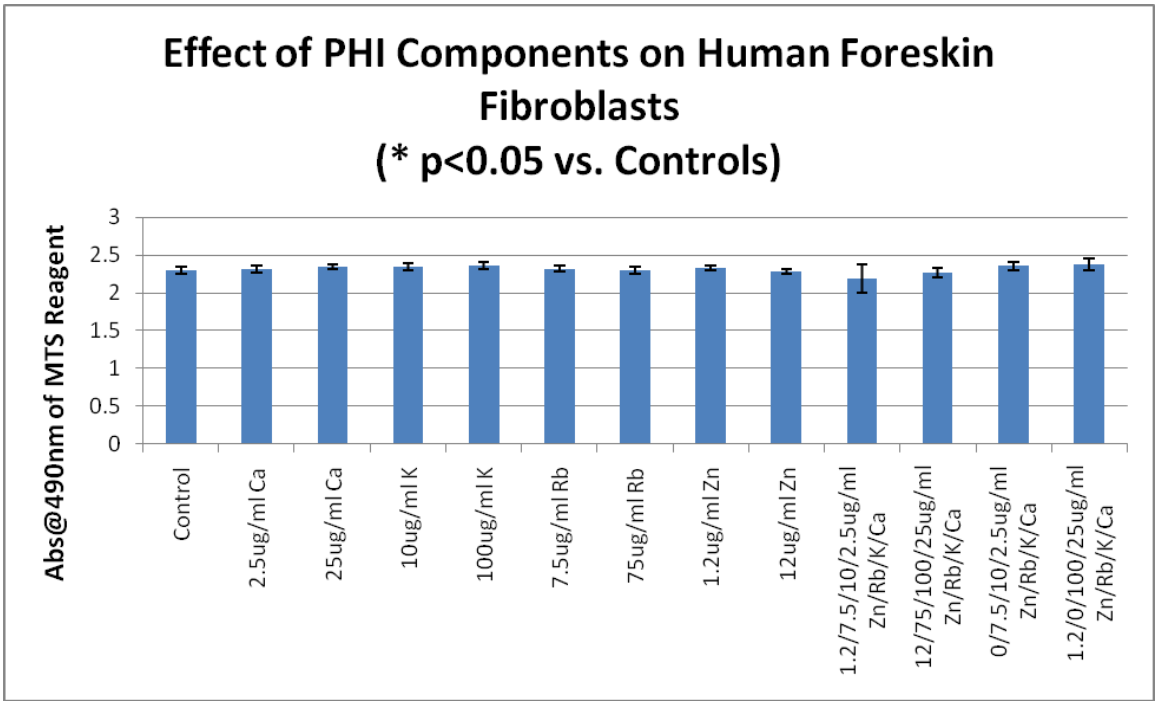

Figure 5. Cytotoxicity of Selected Test Conditions. The absolute absorbances (vs. blanked negative controls) for the various treatment conditions were tested by ANOVA. No cytotoxic effects were indicated for any of the above conditions.

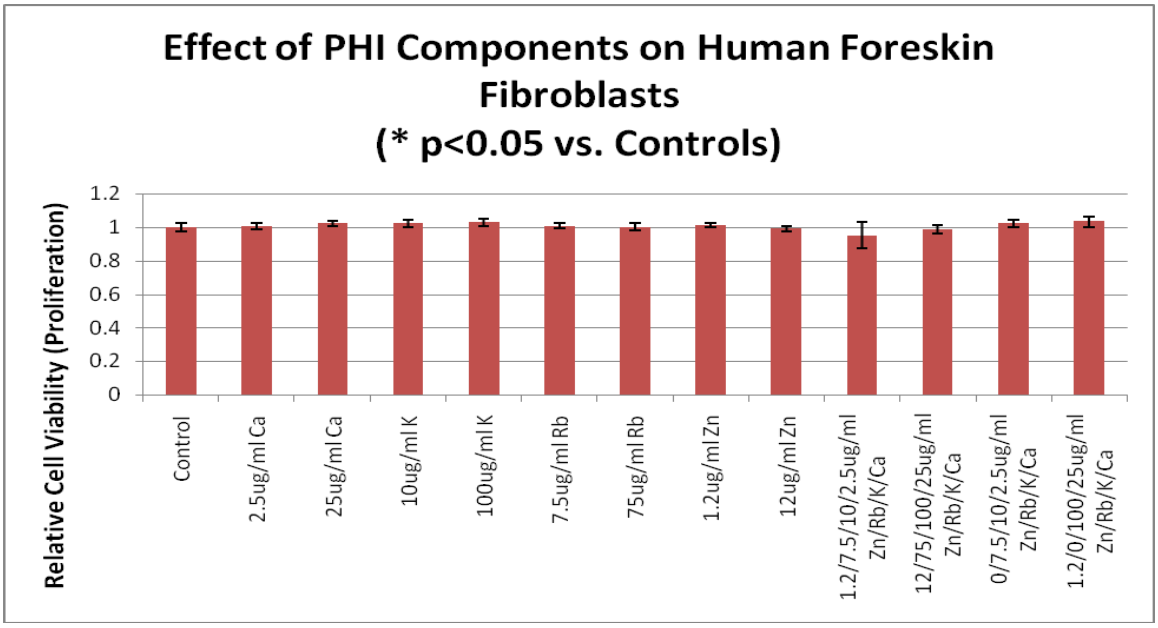

Figure 6. Cytotoxicity of Selected Test Conditions. The relative cell viabilities (vs. blanked negative controls) for the various treatment conditions were tested by ANOVA. No cytotoxic effects were indicated for any of the above conditions.

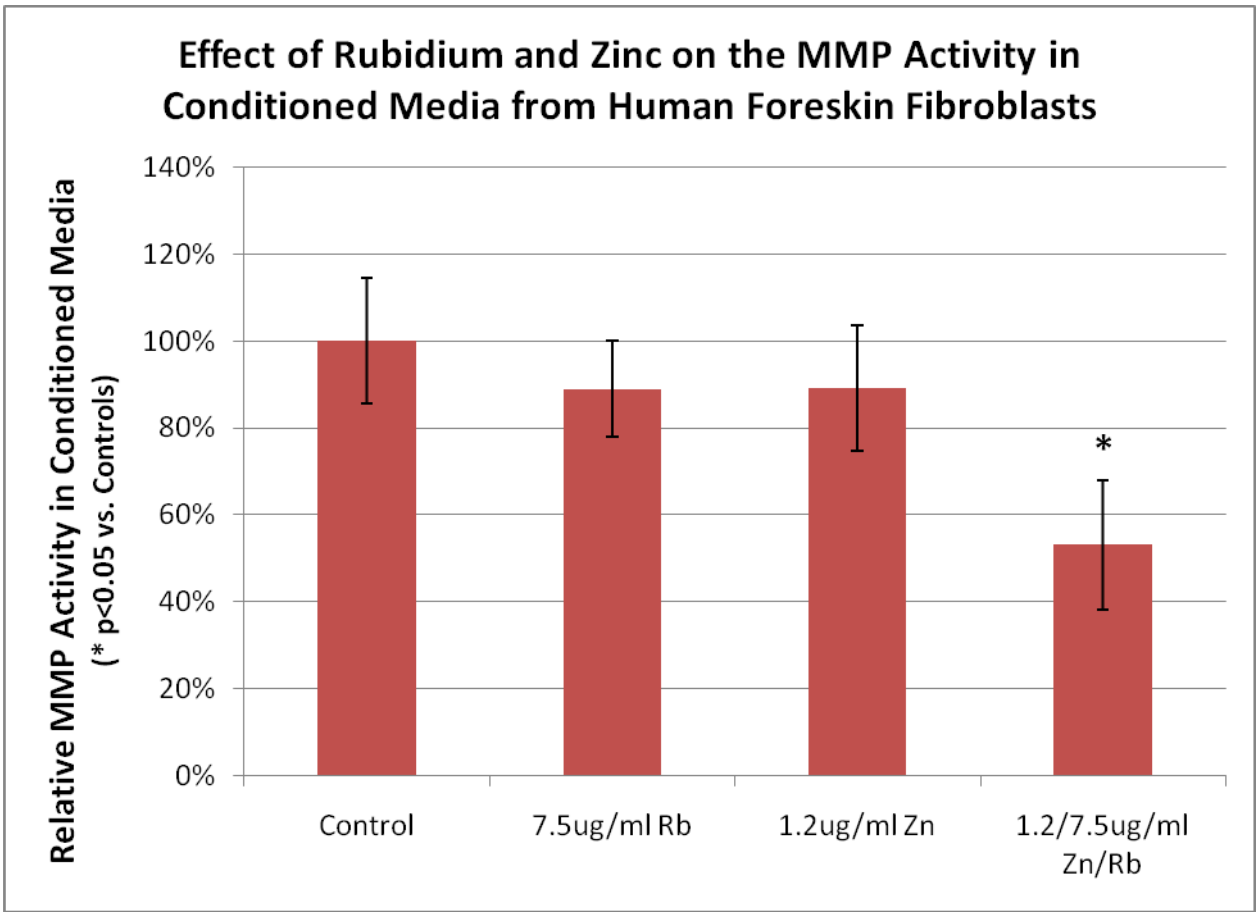

Figure 7. Effect of Rb and Zn on MMP Activity in HFF Cultures. A combination of 1.2ug/ml Zinc and 7.5ug/ml Rubidium effectively reduced MMP expression by 47%±15% (n=5). When cells were treated with either 7.5ug/ml Rb or 1.2ug/ml Zn alone, only slight reductions in MMP activity (11±11 or 11±14, respectively; n=10 each) were observed.