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Corneal Cross-Linking With Riboflavin Using Sunlight

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ABSTRACT

Purpose: To assess whether sunlight might be used to induce a biomechanical stiffening effect in riboflavin-soaked corneas similar to the effect observed in corneal cross-linking using riboflavin and UV-A light.

Setting: Center for Applied Biotechnology and Molecular Medicine (CABMM), University of Zurich, Zurich, Switzerland.

Design: Experimental study.

Methods: Fifty-two porcine eyes were assayed. The concentration of riboflavin in the corneal stroma was estimated using UV-A transmission in a preliminary experiment. Then, the duration of sunlight exposure to achieve a fluence of 7.2/cm² was calculated. Finally, de-epithelialized corneas were divided equally into three groups and soaked with 0.1% (Group Control and 1) or 0.5% riboflavin (Group 2). Eyes from Groups 1 and 2 were then exposed to sunlight. The elastic modulus was calculated as an indicator of stiffness.

Results: Riboflavin concentration in Group B was higher by a factor of 2.8 than Group A. According to live illuminance measurements and stromal riboflavin concentration, the sunlight exposure duration varied between 16 and 45 minutes. Groups 1 and 2 had higher elastic modulus than Control ($P<0.0001$) but did not differ between them ($P=0.194$). The stiffening effect was 84% and 55%, respectively.

Conclusions: Sunlight exposure of *ex-vivo* corneas soaked in both 0.1% and 0.5% riboflavin resulted in increased corneal stiffness. Specifically, 0.1% riboflavin with longer UV-A exposure showed a trend for a greater stiffening effect, which might open new alleys for the use of oral riboflavin and fractioned sunlight exposure as less invasive CXL techniques.

INTRODUCTION

Keratoconus is a progressive corneal ectasia consisting of a focal weakening of the cornea that results in the development of a cone-shaped bulge that usually leads to myopia and irregular astigmatism. If left untreated, keratoconus can result in hydrops, progressive visual blurring and, in extreme cases, corneal perforation. The prevalence of keratoconus appears to vary greatly by geographic region. Keratoconus prevalence is relatively low in Northern Europe and North America, Western Russia and Japan,[1-7] whereas considerably higher in Australia,[8] India,[9] and China,[10] and the Middle East.[11-15]

Keratoconus can develop at any age, but its onset typically starts in childhood or early adolescence, and the disease tends to be more aggressive and progress faster the younger the patient is.[16-18] Part of the reason for this might be that the natural aging process stiffens the cornea, rendering it more resistant to disease progression.[19] Keratoconus has been associated with atopy and eye rubbing, although whether eye rubbing alone is sufficient to cause keratoconus, or simply reveals or exacerbates keratoconus in an already susceptible cornea is still a matter of debate.[20-23]

As the ectasia develops, the weakening cornea becomes less and less able to resist distension by intraocular pressure, leading to the development of a cone-shape protrusion, which brings increasing myopia and irregular astigmatism, resulting in increasingly severe visual impairment.[24] Until the development of corneal cross-linking (CXL) over 20 years ago, the only method of treating keratoconus was keratoplasty.

CXL exploits the photochemical reaction between ultraviolet-A (UVA) light and riboflavin. Riboflavin is photoactivated by UV-A photons, and in the presence of

oxygen, generates reactive oxygen species that can covalently bind molecules in the structural layer of the cornea, the stroma, together, thereby strengthening it with the intent of halting further ectasia progression.[25] Ten-year follow-up data shows its effectiveness in halting keratoconus progression.[26]

Although the CXL is an established method worldwide, there is a treatment inequality issue: many patients living in remote and underserved areas of the world, especially in developing countries, do not have access to the necessary cross-linking devices to perform the procedure safely and effectively. Such devices basically emit 365 nm UV-A light. Notwithstanding, sunlight also includes UV-A wavelengths, so the question arises: could exposure to sunlight effectively cross-link riboflavin-soaked corneas? If so, then this infrastructure issue could be eliminated, which could help prevent visual impairment throughout the world.

Some early experiments dating back more than 25 years have observed that solar irradiation in combination with riboflavin could provide additional resistance to corneas. However, at that time, the aim was to prove the CXL principle; and the riboflavin soaking time and UV irradiation and total energy used were extremely high. More specifically, the authors at the time estimated that each cornea received a light intensity of 85 W/m^2 for 20 minutes with a very long soaking for 45 minutes using riboflavin 0.5%,[27] i.e. far above the limits currently used in daily clinical practice.

In conventional cross-linking, a light source that is almost monochromatic - at 365 nm - is used. This wavelength represents an absorption maximum at the lower end of the riboflavin absorption spectrum, and the light at this wavelength has a higher energy and penetrates deeper into the tissue. The amount of light absorbed in the tissue is also directly related to the concentration of riboflavin at the respective stromal depth. When performing sunlight-induced CXL, one would not use a quasi-monochromatic light source at a short wavelength with high-intensity, but rather use the entire

spectrum of the sunlight. Overlapping the riboflavin absorption curve with the spectrum of sunlight shows that a lot of the cross-linking effect will happen at longer wavelengths, which provide less energy, and also less penetration depth. Therefore, an increase in the relative concentration of riboflavin molecules in the superficial cornea by increasing the riboflavin concentration to 0.5% makes a lot of sense. At the same time, in current clinical practice riboflavin 0.1% is widely used, and hence was also incorporated it into the current study.

The rationale of this study was to explore whether solar irradiation applied at currently clinically used doses would be sufficient to also induce corneal stiffening. In other words, the purpose of this study was to assess the biomechanical effect of sunlight with that of UV-A irradiation on *ex vivo* porcine corneas, using two different riboflavin concentrations, 0.1%, and 0.5%.

MATERIALS AND METHODS

This *ex vivo* study used 52 freshly enucleated porcine eyes with intact epithelium from young adult pigs, between 6 to 8 months of age. All eyes were obtained from the local abattoir and used within 6 hours. In contrast to laboratory-based CXL with a narrow bandwidth light source of 365nm, the light absorption of riboflavin across the sunlight's spectrum (mostly limited to the region 300-500 nm) cannot be assumed constant, see **Figure 1**. Therefore, to compute the overall absorbed energy, which is finally available for the cross-linking reaction, the absolute riboflavin concentration in the stroma needs to be determined experimentally, and the respective extinction coefficient needs to be considered for each wavelength individually.

The study was divided into several phases as follows. Initially, (1) a pre-experiment with 10 corneas was performed to determine the total riboflavin concentration in the corneas to be riboflavin-soaked (Groups A and B). After that, (2) a calculation to

determine how much solar irradiation time is needed to reach a total fluence of 7.2 J/cm² was performed. From this point on, the (3) main experiment was initiated, with de-epithelialized corneas from 42 additional eyes soaked in riboflavin at different concentrations (control Groups, 1 and 2), followed by sunlight exposure (Groups 1 and 2) and subsequent biomechanical characterization.

1. Pre-experiment

Achieved Riboflavin Concentration Determination

Porcine eyes were divided randomly into two groups (Group A and Group B, each with 5 corneas). The epithelium of both corneas was removed, then 0.1% (Group A corneas) and 0.5% (Group B corneas) riboflavin (Vitamin B_2, Streuli Pharma, Switzerland) was dropped onto the corneas every 2 minutes for 30 minutes. The carrier was 400 mOsmol/Lbalanced saline solution in all groups. The corneoscleral buttons were then removed from the eyeball and mounted on a UV-A/B light meter (Sper Scientific LTD, Scottsdale, AZ, USA). A UV-A cross-linking light source (CCL-Vario; Peschke Meditrade GmbH, Zurich, Switzerland) was set to irradiate 365 nm UV-A at an intensity of 18 mW/cm², and the UV-A/B light meter was used to detect the total UV-A transmitted through the corneas in Groups A and B, before and after riboflavin saturation. No cornea control measurements were also taken.

Calculating the Achieved Riboflavin Concentration

In order to derive the stromal riboflavin concentration from the absorption measurements, we used the Lambert-Beer law in combination with previously described absorption characteristics and chemical properties. The Lambert-Beer law describes light intensity *I* as a function of the incident intensity I_0, the penetration depth *th,* the stromal absorption α_C, and the riboflavin-related absorption:

$$I = I_0 \cdot e^{-(C_M \cdot \varepsilon_M \cdot 2.303 + \alpha_C) \cdot thickness},$$

where C_M represents the molar riboflavin concentration and $\varepsilon_M$the molar extinction coefficient. In the absence of riboflavin, the stromal absorption can be calculated from experimental values according to:

$$\alpha_C = \frac{ln\left(\frac{I_0}{I}\right)}{thickness};$$

In the presence of riboflavin, the molar concentration can be calculated from experimental values according to:

$$C_M = \frac{\ln\left(\frac{I_0}{I}\right)}{thickness \cdot \varepsilon_M \cdot 2.303} - \frac{\alpha_C}{\varepsilon_M \cdot 2.303};$$

Where $\varepsilon_M = 10066 \frac{1}{cm \cdot M}$ is the molar extinction coefficient[28] for riboflavin at 365nm, and the factor 2.303 arises from logarithmic conversion. To convert the molar concentration with a unit of mmol/L to the clinically more frequently used riboflavin concentration in percent according to:

$$C_{\%} = M_{riboflavin} \cdot C_M;$$

Where $M_{riboflavin} = 376.34 \frac{g}{mol}$ is the molar mass of riboflavin. A de-epithelialized porcine corneal thickness of 750 µm (850 µm full thickness[29], ~100 µm epithelium) was assumed for the purposes of these calculations. Accordingly, the achieved riboflavin concentration in this pre-experiment (**Table 1**) was 0.0061% and 0.0176% in Groups A and B, respectively.

2. Sunlight Fluence Calculation

Once the riboflavin concentration available in corneal tissue was determined, it was then necessary to calculate the required exposure to sunlight in order to achieve a total sunlight fluence of 7.2 J/cm². For this purpose, both, the full spectral absorption curve of riboflavin and the solar emission spectrum were considered in order to

account for the overall absorbed energy by the full stromal thickness. Furthermore, to account for the large variability in sunlight intensities, live illuminance measurements (luminous-flux per unit area) were conducted to scale the solar emission spectrum accordingly. **Table 2** summarizes the derived necessary irradiation times at different sunlight intensities.

3. Main Experiment: Sunlight Irradiation CXL

Specimens and Preparation

In agreement with earlier literature[29], a CXL-induced stiffening factor of 1.8 was assumed for the sample size calculation. A typical standard deviation of stress-strain measurements was available from earlier studies (0.7 to 1.3 N/m2). Accordingly, a sample size of 7 per group would be necessary to detect CXL-induced stiffening at a power of 0.8 and an α-error probability of 0.05. Applying a higher riboflavin concentration has the potential to change the CXL efficacy. For the purpose of this study, we defined the latter relevant if the induced stiffening is at least 30% higher or lower compared to the standard treatment. Accordingly, with a comparable standard deviation, a sample size of 14 per group is required.

Therefore, in this experimental study, porcine eyes (n=42) were obtained from the abattoir and were randomly divided into 3 equal groups: a control group and two experimental groups were named Group 1 and Group 2, respectively. The corneal epithelium was removed by a hockey knife in all three groups prior to the application of riboflavin.

Riboflavin Application Between Groups

Riboflavin was applied to all corneas via drops administered every 2 minutes for a total of 30 minutes. The corneas of control group and Group 1 eyes received 0.1% riboflavin whereas Group 2 corneas received 0.5% riboflavin.

Sunlight Exposure

After riboflavin instillation, all eyes were fixed in place in a glass Petri dish (diameter=80mm, height=15mm) and covered with a hemispherical quartz glass, transparent to UV-A light, (diameter=50mm, height=32mm; ProQuarz GmbH, Mainz, Germany) to create a wet chamber. The control group Petri dishes were then placed in a light-protected dark room; whereas the Group 1 and Group 2 Petri dishes were placed outdoors to be exposed to sunlight, accompanied by a photometer (DT-1308; CEM, Shenzhen, China) to measure the light intensity of sunlight (**Figure 2**). The sunlight exposure duration was determined by **Table 2** so that each cornea received a total irradiation fluence of 7.2 J/cm².

Biomechanical Characterization

After the removal of the corneoscleral button from the globe, two 5-mm wide full-thickness corneoscleral strips were prepared centrally in the horizontal axis from each eye. Four millimeters from the end of each strip were dedicated to fixation, leaving approximately 11 mm of central corneal strip length. Before stress-strain measurements, all samples were kept in a 400 mOsmol/L balanced saline solution and at a controlled room temperature for 10 minutes. Biomechanical one-dimensional characterization was performed as described previously using a commercial stress-strain extensometer/indenter (Z0.5; Zwick GmbH & Co., Ulm, Germany).[30-32] The biomechanical characterization included elastic testing up to 4.0N standard force. To

calculate the elastic modulus, data from the first cycle was used. The vertical extension was recorded as a function of stress and converted into tensile strain according to the geometrical context, as described previously.[33-35] The stress was calculated from the applied test force, the assumed corneal thickness and specific width of each corneal strip. As two corneal strips of each cornea were evaluated, the tangent elastic modulus of each sample was considered as the average of the two measurements. Homogeneous material properties and no difference between central and peripheral corneal thickness were assumed.

STATISTICAL ANALYSIS

Both the Shapiro-Wilk and Kolmogorov-Smirnov tests were applied to verify the normality of data distribution. Descriptive statistics were described as mean ± standard deviation. ANOVA or Kruskal-Wallis H tests were conducted for continuous variables to analyze the equivalence among all groups, and *post hoc* tests were performed with Bonferroni correction. A *P* value less than 0.05 was the criterion for statistical significance. Statistical analysis was conducted using SPSS version 24 (IBM Corp., Armonk, NY, USA) and the graphs were created in R software version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Figure 3 presents the stress-strain relationship and the elastic modulus as a function of strain for the three different groups. In all corneas, when the strain increased, the elastic modulus raised accordingly. **Figure 4** shows the elastic modulus distribution between 5-10% strain. The mean elastic modulus in Group 1 (3.79 ± 1.35 N/m^2) and Group 2 (3.20 ± 1.24 N/m^2) were both significantly higher than the non-cross-linked control group (2.06 ± 0.70 N/m^2; P<0.0001 and P=0.019, respectively). The stiffening

effect was 84.49% and 55.61% in Groups 1 and 2, respectively. Compared with Group 2, a numerically higher mean elastic modulus was observed in Group 1 but failed to reach statistical significance (*P*=0.194).

DISCUSSION

Approximately 5% of solar radiation is UV radiation, 95% of which is composed of UV-A (i.e., has a wavelength of 315-400 nm).[36] UV-A is an essential component of CXL.[25] In the present study, significantly increased biomechanical strengthening was observed in both experimental groups, suggesting that sunlight exposure in combination with riboflavin saturation of the corneal stroma is sufficient to cross-link the cornea to an extent where increases in corneal biomechanical strength can be detected.

Before the classic “Dresden protocol” method of performing CXL was introduced to the clinic,[37] Spoerl et al.[27] analyzed the stress-strain relation of porcine corneas after they underwent CXL using 0.5% riboflavin solution and different wavelengths of UV light (254 nm and 365 nm), blue light (436 nm) and sunlight. After performing stress-strain analyses on the post-cross-linked corneas, they found that only the 365 nm and sunlight-exposed corneas showed obvious significant increases in biomechanical strength. When the strain was 5%, the stiffening effect approached 200% in the 365 nm UV-A-treated group and 80% in the sunlight-treated group. However, in the aforementioned study by Spoerl et al., twice as much of the stiffening effect was observed after CXL with sunlight than in the present study. The different riboflavin soakage times and the different exposure times used might account for these disparities. While our riboflavin application used the same protocol and solution that is currently used clinically, Spoerl et al. used 0.5% riboflavin and soaked the

cornea for 45 minutes, which may have affected the stiffening effect. Another reason may be the difference in sunlight exposure and total irradiance fluence between both studies: the Spoerl et al. study performed 120 minutes of sunlight exposure with an undetermined – although likely much higher – applied fluence.

Stromal riboflavin concentration may be a factor regarding how effective sunlight can cross-link the cornea: Group 1 corneas tended to be stiffer (and therefore, it is reasonable to assume, more effectively cross-linked) than Group 2 corneas, despite a lower riboflavin concentration being used. We speculate that the lower riboflavin concentration may have helped with oxygen diffusion and replenishment in the stroma through a longer irradiation time. In two previous *ex vivo* studies, Wollensak et al.[29] found that the standard Dresden protocol stiffened human corneas more than porcine corneas; Kling et al. demonstrated that thin corneas were disproportionally stiffened more by CXL than thicker corneas,[38] and it is worth noting that porcine corneas are thicker than human corneas.[39] As a consequence, we could assume that the here observed sunlight-induced increase in biomechanical properties obtained by CXL might be even greater in human corneas.

The combination of low riboflavin concentrations with sunlight irradiation, resulting in stiffer corneas, opens the possibility of combining oral riboflavin with long-term fractionated sunlight exposure (i.e., days instead of minutes) in order to strengthen weakened – or even predisposed – corneas and help prevent corneal ectasia progression. The first mention in this regard was made in 2018 in an ARVO poster by Schaeffer and Jarstad *et al* suggesting that ingestion of high doses of oral riboflavin and ambient UV light can result in corneal flattening, which the authors hypothesized was due to sunlight-induced cross-linking.[40] The authors' suggestion was made after

observing corneal flattening between zero and a maximum of 2.1 D in the 6 eyes of the 3 patients included in the analysis, of which 2 were monitored for 6 months and one for only 1 month after taking riboflavin 400mg or 500 mg BID or TID.[40] The paper mentioned a new IRB-approved study that would be underway at the time,[40] but to the best of our knowledge, such results have not yet been reported.

Several points need to be discussed on such an impressive observation. Even if the disadvantages of epithelial removal – such as postoperative pain and risk of infections[41] – could be avoided by oral riboflavin administration with fractional exposure to sunlight, we would still need to assess whether the integrity of the epithelium, which acts as a barrier to sunlight exposure and oxygen diffusion, could limit an effective clinical response.[42] Apart from the epithelial and oxygen barrier, the concentration of stromal corneal riboflavin reached by oral intake is potentially lower than that usually found when riboflavin is applied to the exposed corneal stroma. Finally, unlike our present study where corneas were directly exposed to UV irradiation, this is clearly not feasible in human eyes – which would therefore also limit a possible clinical effect, at least in the short term. In this sense, it seems surprising that the patient with the maximum flattening among the only three reported by Schaeffer and Jarstad et al had a flattening of about 2 D in one eye after only 1 month of oral riboflavin intake.[40] We believe that before envisaging any comparisons, it is necessary to bear in mind all these potential limitations being overcome. To verify all these hypotheses, *in vivo* studies are being carried out to evaluate both the riboflavin bioavailability, the specific stromal riboflavin concentration, and the stiffening effect resulting from this interaction in live animals.

A limitation of the present study was the use of porcine eyes instead of human corneas. As mentioned before, the thicker porcine corneas may not only affect oxygen diffusion, but also stromal riboflavin concentration and UV-A transmission,

resulting in a potentially lower accuracy of simulating the human eyes. Furthermore, it is also necessary to reinforce that in the present study the corneas were subjected directly to solar irradiation, which would be unfeasible in human eyes, precisely because looking directly at the light source (or the sun) is not a possibility. On the other hand, much of the cross-linking technology has evolved through the study of porcine eyes, and so far, the experimental and clinical correlation has been consistent.[31, 32, 43-50] Therefore, with all due limitations, the findings may to some degree match the biomechanical response in human eyes. A potential limitation that one could envisage would be the hydration of the samples submitted to sunlight. We are aware that hydration is a fundamental factor, and therefore this variable was controlled from the beginning and in all stages of the study in an optimal way, as mentioned in the methods. Additionally, Hatami-Marbini et al showed that changing the hydration status prior to cross-linking treatment did not significantly alter the amount of biomechanical improvement if tensile properties are measured at similar hydration states.[51] Moreover, Wollensak demonstrated that hydration variations in the cornea do not reduce the stiffening effect observed in cross-linking procedures.[52] Therefore, since hydration was optimally controlled in the present study, we do not believe that this impacted any outcome presented. Finally, a weakness of the study was the inability to analyze the corneal healing after sunlight-induced CXL, since solar radiation comprises a wide spectrum of visible and ultraviolet wavelengths, as opposed to a single UV-A wavelength, and this might produce a different healing response and thus affect the post-CXL properties of the cornea. As mentioned, *in vivo* studies are being carried out to evaluate such a response.

In summary, sunlight exposure of *ex vivo* corneas soaked in both 0.1% and 0.5% riboflavin resulted in increased corneal stiffness. Group 1 (0.1% riboflavin with longer sunlight exposure) had a trend for to greater stiffening effect, likely due to greater

oxygen availability. This might open new avenues for the use of oral riboflavin and fractioned sunlight exposure as less invasive CXL techniques. *In vivo* studies are currently underway to confirm this hypothesis.

Supplementary Material - http://links.lww.com/JRS/A931

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Table 1: UVA Transmission (μW/cm²) in 8mm-Diameter Using Irradiation 18mW/cm². C_M represents the molar riboflavin concentration (mmol/L) and $C_{\%}$ represents the riboflavin concentration in percent, in Groups A and B. Accordingly, the achieved mean riboflavin concentration was 0.0061% and 0.0176%, respectively.

Porcine cornea number	Total Transmission (no cornea)	Before riboflavin (epi-off, cornea without soaking)	**Group A** (epi-off and 0.1% riboflavin soaking)	**Group B** (epi-off and 0.5% riboflavin soaking)
1	15500	10690	4956	2612
2	15500	10550	4920	4186
3	15500	10333	5380	2525
4	15500	10749	5495	3502
5	15500	10135	6003	2915
UVA transmission (mean ± SD)	15500.0±0	10491.4±255.6	5350.8±443.98	3148.0±695.1
C_M mean			1.63E-04	4.68E-04
C_M upper			2.13E-04	6.11E-04
C_M lower			1.17E-04	3.53E-04
$C_{\%}$ mean			**0.0061**	**0.0176**
$C_{\%}$ upper			0.0080	0.0230
$C_{\%}$ lower			0.0044	0.0133

Table 2: Exposure duration to sunlight in a total fluence of 7.2 J/cm²

	0.1% Riboflavin & Epi-off (Estimated stromal concentration: 0.0061%)	0.5% Riboflavin & Epi-off (Estimated stromal concentration: 0.0176%)
Intensity (klux)	Irradiation Time (minutes)	Irradiation Time (minutes)
3	1398.07	593.29
4	1048.55	444.97
5	838.84	355.97
6	699.03	296.65
8	524.28	222.48
10	419.42	177.99
15	276.98	117.54
20	208.22	88.36
30	139.14	59.05
40	104.11	44.18
50	83.41	35.40
60	69.57	29.52
70	59.55	25.27
80	52.15	22.13
90	46.38	19.68
100	41.70	17.70
110	37.93	16.10

FIGURE LEGENDS

Figure 1. Overlapping region of the solar emission spectrum and the riboflavin absorption spectrum. The whole region between approx. 300 to 500 nm contributes to the CXL reaction and needs to be considered.

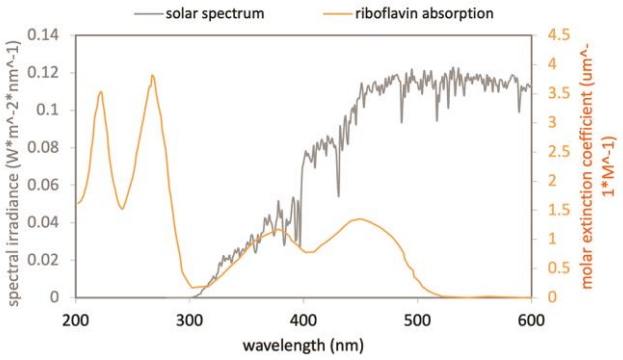

Figure 2: Porcine eyes were placed outdoors to receive sunlight irradiation; a photometer was set aside to measure live sunlight illuminance.

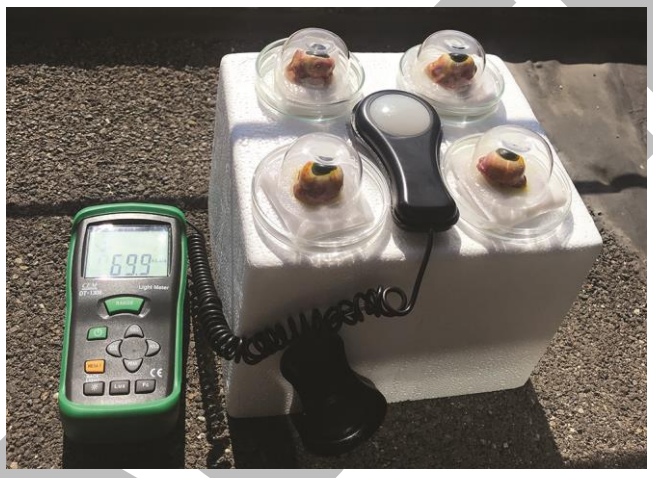

Figure 3: Elastic Modulus-strain data for all the groups under different strain.

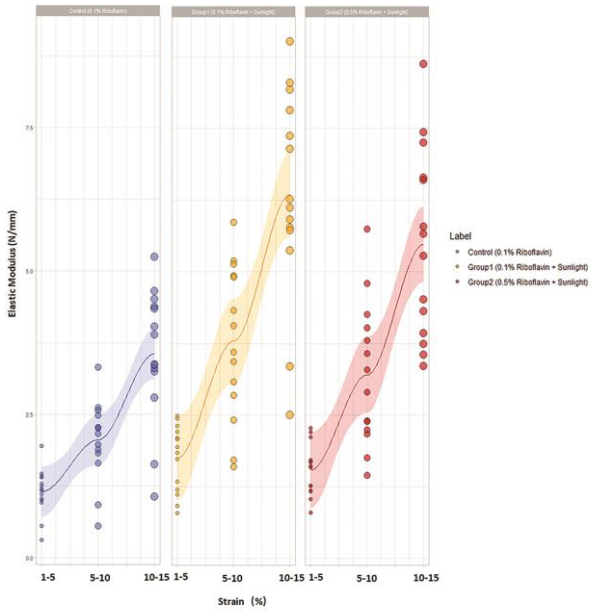

Figure 4: Elastic Modulus Distribution Between 5–10% strain.