An innovative method for rapid detection of urinary oxalate in goats with urolithiasis

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Short Report

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Abstract

Background: Oxalate is a natural and abundant metabolic by-product; as a highly oxidized organic compound with powerful chelating activity, in high concentrations it can cause morbidity and mortality in both animals and humans. Elevated levels in urine (i.e., hyperoxaluria) have been found to correlate with several human diseases, especially urolithiasis or kidney stone disease (KSD)- a disease also prevalent in goats. Current methods for measuring oxalate are highly technical, cumbersome, and time-consuming, which often forces clinics to utilize expensive diagnostic laboratories. Therefore, in this study, we designed an innovative device, Oxalometer, to measure urinary oxalate in goats.

Results: The results indicate that the Oxalometer performed as accurately as the standard commercially available test. The Oxalometer was able to measure higher levels of urinary oxalate in goats with urolithiasis compared to non-urolithiasis goats. The data demonstrate the accuracy and sensitivity of the Oxalometer.

Conclusions: This proof-of-concept study supports the future application of the device in determining on-the-spot oxalate levels in patients and brings kidney stone prevention to point-of-care practice for ruminants.

Background

The goat industry in the US has grown significantly in the past decade (1), with high demand attributed to several emerging factors, such as population growth of goat meat-consuming countries, increase in health conscious meat consumption based on free range, lean, organic, and sustainable food sources, and other factors including dairy production (especially milk and cheese), manufacturing (e.g., angora and cashmere fiber)(2), entertainment (e.g., 4-H activities)(3), and biotechnology (models of human disease, commercial antibody production)(4) (5). Notably, goats are browsing animals that feed on shrubs, bushes, and trees, demonstrating a highly adaptable digestive behavior (6) and, as free-ranging animals, goats spend approximately 30% of the day feeding surrounding vegetation (6, 7). These factors, specifically the consumption of plants containing high quantities of potassium and sodium oxalates, are the leading causes for urolithiasis in goats via oxalate toxicity (6, 8, 9). This often results in obstructive urolithiasis, or the inability to excrete urine due to obstruction of urinary outflow tract by calculi (10, 11), which is one of the most prevalent urinary diseases associated with goats especially in young and castrated males. The pathophysiology of obstructive urolithiasis is complex and multifactorial, as it is speculated to stem from the dynamic interactions of nutritional, physiological, and management-related factors. These etiological factors prescribe the types of obstructive calculus, specifically calcium phosphate (apatite), magnesium ammonium phosphate (struvite), or calcium oxalate (12).

Although oxalic acid is a natural and abundant by-product of metabolism, it is also a highly oxidized organic compound with a potent chelating activity that, in high concentrations, can cause death in both animals and humans due to its corrosive effects (13–15). Since mammalian species cannot break down

oxalic acid, it must be excreted via urine; high levels of oxalic acid in the urine (i.e., hyperoxaluria) have been found to correlate with a number of human diseases, especially calcium-oxalate kidney stone disease (CaOx KSD), a disease also prevalent in goats (6, 7). The current oxalate test used in human medicine is relatively expensive, utilizing costly equipment performed by skilled technicians; it is time-consuming with a longer turnaround time. Since the current test is not a point-of-care test, monitoring or treatment plans are challenging to implement for patients at the time of visit. This study developed an Oxalometer device that can measure oxalate levels in urine within two minutes. The Oxalometer exhibited similar accuracy and sensitivity as a commercially available test. Furthermore, we found that goats suffering from urolithiasis had higher levels of urinary oxalate than non-affected goats. This proof-of-concept study supports the future application of the device in determining on-the-spot oxalate levels and brings kidney stone prevention to point-of-care practice.

Methods

Animal recruitment and sample collection.

Owners of goats admitted to the University of Florida Large Animal Hospital were asked if they would participate. The animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida. Clinical examination upon hospitalization consisted of a complete physical examination and diagnostic imaging procedures. Transabdominal ultrasonography was performed with a 3.5 MHz sector probe to confirm bladder enlargement and a diagnosis of obstructive urolithiasis. The urine samples were collected from goats with obstructive urolithiasis (n = 12) and goats without urinary tract disorders (n = 15) either by free catch or ultrasound-guided cystocentesis as presented in Table 1. Uroliths obtained as part of the treatment in some goats or necropsy examination of all goats presenting for obstructive urolithiasis were collected. Recovered uroliths were submitted to the Minnesota Urolith Center in St. Paul, Minnesota, where uroliths were screened by optical crystallography and infrared spectroscopic for confirmation of urolith composition.

Table 1
Goats' health condition

Goat #	Reason for admission	Diagnosis	UA collection
1	Pregnancy check, abortion	Fetal Death	Free catch
2	Pregnancy check	Abortion	Free catch
3	Pneumonia, urolith in 2013	Lung disease/ Urolithiasis	Free catch
4	Urolithiasis	Urolithiasis	Free catch
5	Pneumonia	N/A	Free catch
6	Pregnancy check	Hernia-incisional	Free catch
7	Mastitis	Mastitis/ Urolithiasis	Free catch
8	Pruritis, eosinophilia	Diarrhea/ Urolithiasis	Free catch
9	Pemphigus	N/A	Voided
10	Dystocia	Dystocia	Free catch
11	Pregnancy check	Pregnancy	Free catch
12	Pregnancy check	Pregnancy	Free catch
13	Pregnancy check	Pregnancy	Free catch
14	Pregnancy check	Pregnancy toxemia	Free catch
15	Urolithiasis	Urolithiasis	Cysto
16	Abscess	Abscess	Free catch
17	Dysuria	Urolithiasis	Free catch
18	Urolithiasis, pyelonephritis	Urolithiasis	Free catch
19	Urolithiasis	Urolithiasis	Free catch
20	Urolithiasis	Urolithiasis	Free catch
21	Diarrhea	Diarrhea-acute	Free catch
22	Urinary straining	Urolithiasis	Free catch
23	Urinary blockage	Urolithiasis	Free catch
24	Possible urolithiasis or colic	Colic	Free catch
25	Regular check	N/A	Free catch
26	Urinary straining	Urolithiasis	Free catch
28	Not growing properly	Parasites	Free catch

Design and Stereolithographic Apparatus (SLA) 3D printing of the oxalometer filtration cartridge.

The oxalometer filtration cartridge was designed using Trimble Sketchup 3D design software. The column design interfaced a slanted grate with the sidewall of a standard 1.5 mL polystyrene disposable 1 cm path length cuvette (ThermoFisher, USA). In essence, the design employed the cohesive natural properties of water and adhesion to the cuvette surface to uniformly and consistently draw fluid through the filter. 3D printing was achieved using the Form 1 + SLA 3D Printer (FormLabs, USA), which uses a blue light laser (305 nm) to fuse resin layers to form a water-tight object. STL files from Sketchup were imported into the proprietary PreForm software. Positioning and support structures were automatically optimized by the software before printing, and the same settings were used on each following print. The build surface (150 mm x 150 mm) was capable of printing 29 filtration columns simultaneously after optimization. Clear Resin (#GPCL02) was filled into the reservoir tank, and 3D printing was initiated at a print resolution of 200 microns. Completed prints were scraped off the build stage, rinsed in isopropyl alcohol by repeatedly dunking for 2 minutes, and submerged for 5 minutes. The prints were air dried for 5 minutes and cured afterward using a long wavelength UV light for 30 minutes. Cured prints had the supports trimmed off using end-cut pliers. Columns were treated with Elmer's spray glue, and glass fiber (Whatman GF-D) filter paper was adhered to the inside bottom face of the columns. 150 mg/mL of 100 mesh activated charcoal in water were mixed, and 1 mL was pipetted into each column and allowed to drip dry to create the final filtration column (Fig. 1).

Determining urinary oxalate using 3D printed oxalometer.

Succinate buffer was prepared by mixing 26.8 mL of ddH_2O, 5 mL of 500 mmol/L of succinic acid (pH 3.8), 0.6 mL of 100 mmol/L of p-Dimethylaminobenzaldehyde (DMAB), and 0.6 mL of 100 mmol/L of 3-Methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH). 4 mL of 0.2 U/mL enzyme mixture containing oxalate oxidase and horseradish peroxidase (Trinity Biotech, USA) was added to the succinate buffer. The mixture was vortexed, and 500 µL was pipetted into empty cuvettes, then capped and stored in a -80°C freezer until use. Cuvettes were thawed for approximately 15 minutes, caps were removed, and 50 µL of urine was added to each oxalometer filtration charcoal cartridge. Cuvettes were centrifuged (1000 rcf, 30 seconds), then filter cartridges were removed, and cuvettes were re-capped; cuvettes were incubated at 37^0C for 15 minutes. Immediately after, the results were read at 590 nm on the Tecan M200 Pro Spectrophotometer with a cuvette adapter. All samples were measured in duplicate, and the experiment was repeated three times.

Determining urinary oxalate using a commercial kit.

The colorimetric detection system was used to measure urinary oxalate per the manufacturer's instructions (Oxalate kit, 591-C, Trinity Biotech, USA). Briefly, oxalate reagents A, B, and the sample diluent were suspended with 10 mL, 2 mL, and 100 mL, respectively. Standard concentrations of 0.357 mM oxalate and 0.5 mM oxalate were prepared using potassium oxalate in normal simulated urine (Carolina,

695955). For each sample, a 1:1 ratio of urine: sample diluent was utilized and stabilized at a pH between 5 and 7 prior to being transferred to a charcoal tube. Each sample was vortexed, then centrifuged (1500xg, 5 minutes); 100 µL of sample Reagent A was added to each well, followed by 5 µL of standards, sample urines, or water (negative control), to respective wells. 10 µL of Reagent B was added to each well and incubated (room temperature, 5 minutes), then absorbance at 590 nm was read on the Tecan M200 Pro Spectrophotometer. All samples were measured in duplicate, and the experiment was repeated three times.

Statistical analysis.

GraphPad Prism 6 software was used for all statistical analyses. Differences between the two groups (urolithiasis v non-urolithiasis goats) were determined by an unpaired two-tailed parametric Mann-Whitney test, and the median was reported. Statistical significance was considered if $p < 0.05$ in all analyses.

Results

Evaluating the accuracy of the Oxalometer.

The Oxalometer has a few components that could compromise the accuracy of the device. For example, the filtration charcoal cartridge may impede the flow of the liquid sample by gravity, or the flow rate could affect the colorimetric reaction in the cuvette. Therefore, it is imperative that we determined the reliability and accuracy of the Oxalometer against a commercially available standard test (Trinity oxalate kit). Simulated urine (Item # 695955, Carolina Biological, USA) was spiked with defined concentrations of oxalate (0.25, 0.5, 1.0 uM). Absorbance for each concentration was determined using the Oxalometer and Trinity oxalate kit. As presented in Fig. 2, the Oxalometer showed a direct relationship between urinary oxalate concentration and absorbance, as expected. In comparing the two testing platforms, the slope of the Oxalometer was 0.08917 [0.06655, 0.1118] and y-intercept was 0.1217 [0.1088, 0.1347], whereas, the Trinity oxalate test yielded the 95% confidence interval with the slope of 0.07098 [0.03858, 0.1034] and the y-intercept of 0.1424 [0.1239, 0.1610]. Furthermore, the coefficient of determination, or R-squared, of the Trinity oxalate kit was 0.7044, while the R-squared of the oxalometer was slightly higher at 0.8853. The result indicates that the Oxalometer performed similar accuracy to that of the standard for oxalate measurement.

Measuring goat urinary oxalate using the Oxalometer.

As demonstrated, the Oxalometer performed as well as the commercial standard with known oxalate concentrations in simulated urine. To further evaluate the sensitivity and accuracy of the Oxalometer, clinical urinary samples were utilized. Urinary oxalate levels in goats with obstructive urolithiasis were compared to urinary oxalate levels in non-urolithiasis goats. Using the Trinity kit, measurement of urinary oxalate in goats with urolithiasis was significantly elevated above that of non-urolithiasis goats (Fig. 3A and 3B; mean absorbance in non-urolithiasis goats: 0.551 ± 0.040, urolithiasis goats: 0.797 ± 0.068, p =

0.0144, mean concentration in non-urolithiasis goats: 5.762 ± 0.563, urolithiasis goats: 9.228 ± 0.954, p = 0.0144). Next, we sought to determine the accuracy of the oxalometer with goat urines. As presented in Fig. 3C, there was a significant difference between the urinary oxalate in non-urolithiasis and urolithiasis goats (mean absorbance in non-urolithiasis goats: 0.050 ± 0.008, urolithiasis goats: 0.126 ± 0.018, p = 0.0109). The mean concentration was 4.254 µM ± 0.772 for non-urolithiasis goats and 10.554µM ± 1.401 for urolithiasis goats, p = 0.0028 (Fig. 3D). The result indicates that the Oxalometer could accurately measure urinary oxalate in clinical samples.

Discussion

Oxalate is a natural by-product of metabolism that cannot be broken down by mammals instead of excreted through urination. Hyperoxaluria can result in kidney stone disease (KSD) or urolithiasis. Often clinics are forced to utilize expensive diagnostic laboratories to measure urine oxalate because current methods are highly technical and cumbersome. The standard of care oxalate methods (Trinity Biotech, MilliporeSigma) which the diagnostic laboratories often use, involve expensive instruments, such as incubators, mixers, spectrophotometers, and can take up to three hours to complete and additional days to obtain the result. Therefore, in this study, we designed a rapid and innovative device called Oxalometer as an alternative to measuring urinary oxalate. We first validated the measurements of urinary oxalate levels in simulated urine spiked with defined concentrations of oxalate. Both the standard commercial kit and the Oxalometer exhibited a direct relationship between oxalate concentrations and absorbance; specifically, as the oxalate concentration increased, the absorbance also increased. Interestingly, the R-squared of the Oxalometer device was higher than that of the commercial assay, suggesting that Oxalometer has a better fit for the data. Furthermore, using clinical goat urines, the Oxalometer reproduced the same results as the commercial assay, suggesting that our new device can accurately and consistently detect urinary oxalate levels.

Data of quantitative mineral analysis of uroliths obtained from 1981–2007 of 941 ruminants by the Minnesota Urolith Center indicated that the majority of ruminants were diagnosed with calcium carbonate uroliths and 1.7% with calcium oxalate. Although with a small sample size, the prevalence of calcium oxalate uroliths was highest in bison, bongo, and caribou. Within this cohort, 42% and 0.8% of goat uroliths were composed of calcium carbonate and calcium oxalate, respectively. Calcium carbonate stones occur more commonly in animals fed forage or grass, calcium oxalate stones are associated with ingestion of oxalate-containing plants, struvite stones (magnesium ammonium phosphate) are associated with consumption of grain, and silica stones are more common in the Western United States and Canada where grasses have higher silica concentrations. Urine pH also plays a vital role since alkaline pH favors the development of calcium carbonate, struvite, and apatite stones. Currently, there is a lack of information that demonstrates the direct correlation between urinary oxalate levels with calcium oxalate stones in wild or domestic animals. However, human epidemiologic studies have clearly shown the importance of urinary oxalate excretion on kidney stone risk. A prospective study examining the relationship between oxalate intake and initial kidney stone development in the Health Professionals Follow-up Study (n = 45,985 men), the Nurses' Health Study I (n = 92,872 older women), and the Nurses'

Health Study II (n = 101,824 younger women) reported that higher dietary oxalate was associated with increased stone risk in men and older women (16). When researchers focused on those with a recurrent stone disease in this cohort (~ 6,000 individuals), stone formation risk significantly rose with increasing urine calcium and oxalate excretion (17). Of these two factors, Siener *et al*. demonstrated that increased oxalate excretion, not calcium, was the major urinary risk factor for relapse during follow-up with recurrent calcium oxalate stones (18). This finding was further corroborated by *in-vivo* and *in-vitro* studies that showed small increases in urinary oxalate could have a confounding effect on the factors which influence nucleation, crystal growth, and aggregation of calcium oxalate in urine (19).

Increased awareness of oxalate-based diseases and the prevalence of urolithiasis in animals and humans have catalyzed a need for simple, rapid assays that provide temporal-based monitoring of oxalate levels in urine and/or blood. Current methods for determining oxalate, however, are relatively complex, labor-intensive, and require specialized equipment in the laboratory setting. In general, a spectrophotometer is needed to measure the intensity of indamine dye at 590 nm as an end-product of oxalate, 3-methyl-2-benzothiazolinone hydrazone (MBTH), and 3-dimethylaminobenzoic acid (DMAB) catalyzed by oxalate oxidase and peroxidase (20–22). A modified HPLC spectrophotometric detector can be used to improve sensitivity (23), while ion chromatography can be used to determine oxalate and multiple other ion concentrations (24, 25). The current oxalate test is relatively expensive and performed by skilled technicians using costly equipment. With turnaround times between 3–7 days, it is not a point-of-care test, resulting in inpatient treatment plans that are difficult to prescribe and implement. While testing for oxalate would seem an essential standard of care in stone disease, urinary oxalate testing is not performed routinely.

Conclusions

In this study, we developed Oxalometer device to determine the level of urinary oxalate accurately. The critical feature of the device is the simplicity in manufacturing and application and its convenience and timeliness to run in-clinic or at home. Routine monitoring would enable animal owners and veterinarians to become aware of the animal's oxalate levels and to take measurements quickly to prevent or reduce the possible recurrence of kidney stones or other oxalate-related diseases through changes in diet and fluid intake. One major drawback of the study is the small sample size. Therefore, additional studies with larger cohorts will be needed to validate the results furthers. Validation of this technology in other species, including dogs, cats, and humans, could also revolutionize point-of-care oxalate testing.

Abbreviations

CaOx KSD: calcium-oxalate kidney stone disease

SLA: Stereolithographic Apparatus (SLA)

DMAB: p-Dimethylaminobenzaldehyde

MBTH: 3-Methyl-2-benzothiazolinone hydrazone hydrochloride hydrate

KSD: Kidney stone disease

HPLC: High pressure liquid chromatography

Declarations

Ethics approval and consent to participate

The animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida.

Authors' contributions

AW developed the device using the 3D printing of the device. TE, PS, AV, and ST carried out the experiments and data analyses. SR recruited patients. TE, SR, AV, and CN conceptualized the study and prepared the manuscript. The authors read and approved the final manuscript.

Competing financial interests

CQN is the founder and Chief Executive Office AP LifeSciences. CQN holds a patent on the compositions and methods for monitoring oxalate (PCT/US2012/024164).

Availability of data and materials

All data generated or analysed during this study are included in this published article.

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Table

Table 1: Goats' health condition

Goat #	Reason for admission	Diagnosis	UA collection
1	Pregnancy check, abortion	Fetal Death	Free catch
2	Pregnancy check	Abortion	Free catch
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Figures

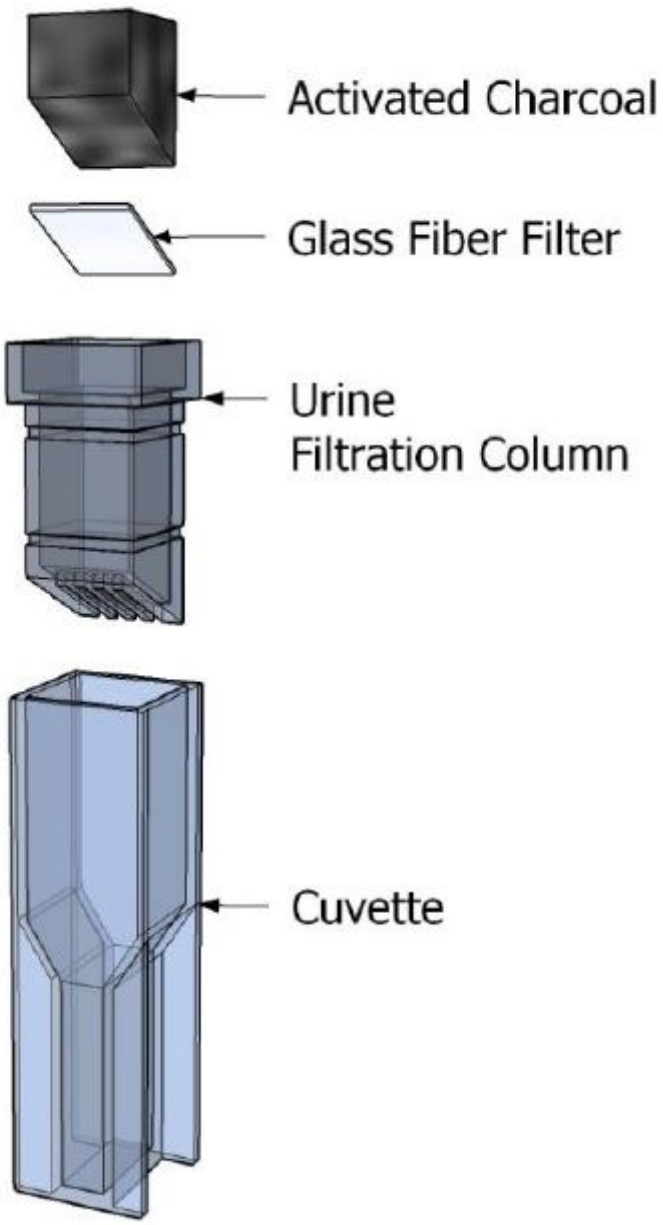

Figure 1

Oxalometer assembly schematic: Using 3D printing, the Oxalometer was designed with a urine filtration unit coupled with an enzyme-dye preloaded cuvette to allow for a simple and quick urinalysis. The Oxalometer filtration cartridge was developed using the Trimble Sketchup 3D design software, while the column design consisted of a standard 1.5 mL polystyrene disposable 1cm path length cuvette interfaced with a slanted grate. Glass, the fiber filter paper was adhered to the inside bottom face of the columns, and mesh activated charcoal was added into each column to create the filtration column. This device employs the properties of cohesion and adhesion to the cuvette surface to homogenously draw fluid through the filter.

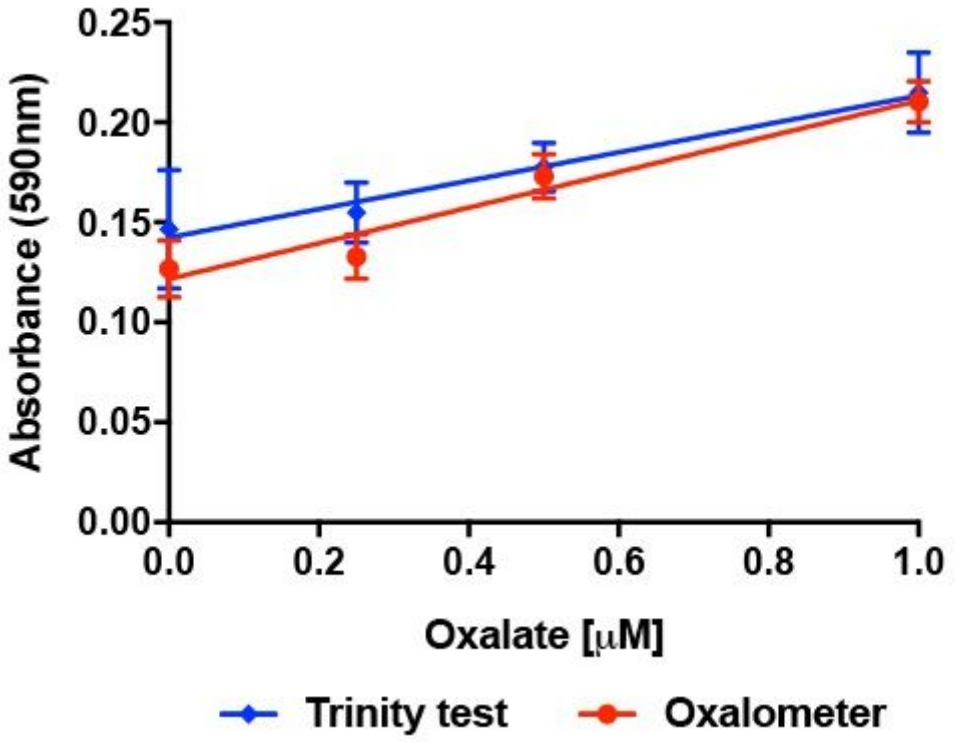

Figure 2

Evaluating the accuracy of the Oxalometer: In comparison to the standard of care test, the Oxalometer also demonstrated a direct relationship between urinary oxalate concentration and absorbance. The slope of the Trinity oxalate kit was of 0.07098 [0.03858, 0.1034] and the y-intercept of 0.1424 [0.1239, 0.1610] while the slope of the Oxalometer was 0.08917 [0.06655, 0.1118] and y-intercept was 0.1217 [0.1088, 0.1347]. The coefficient of determination of the Trinity oxalate kit was 0.7044, while the R-squared of the Oxalometer was higher at 0.8853. Mann-Whitney t-test - *P<0.05, **P<0.005

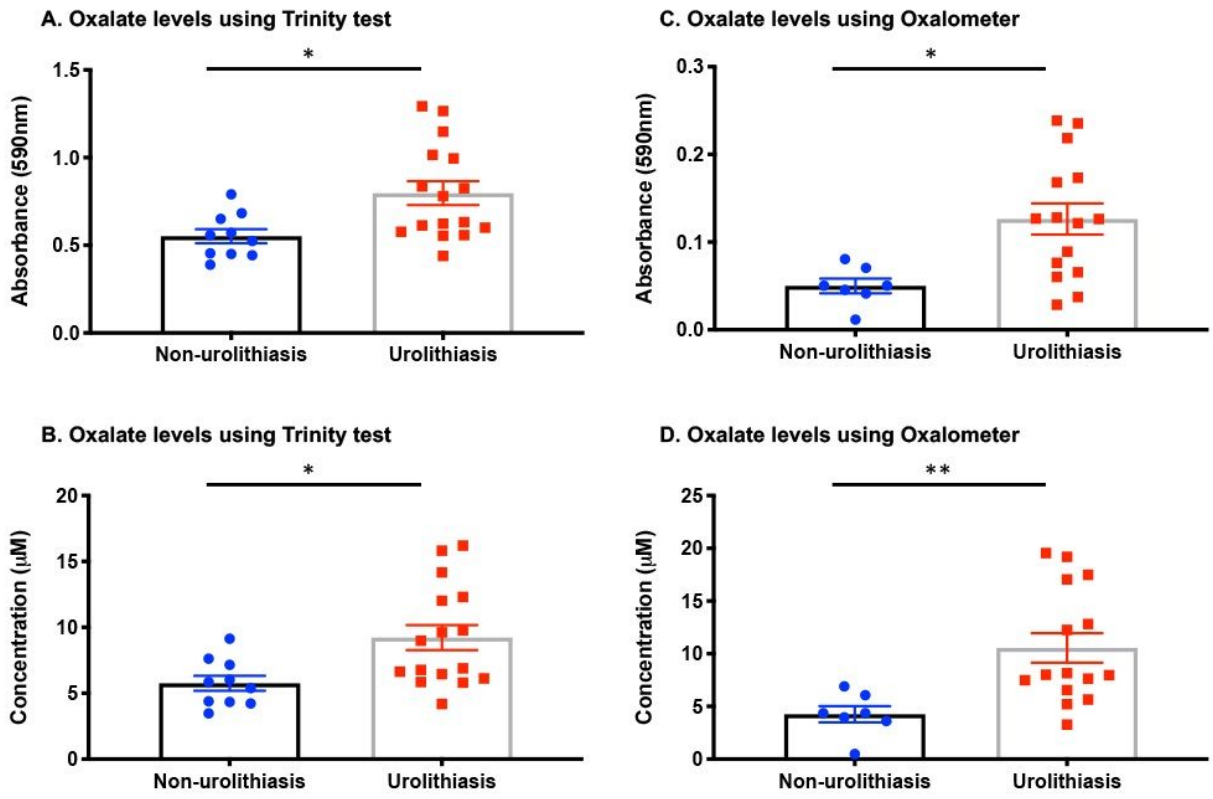

Figure 3

Goats suffering from urolithiasis have higher levels of oxalate in urine. Urinary oxalate in urolithiasis goats was significantly elevated above that of non-urolithiasis goats when using the Trinity oxalate kit (Figure 3A and 3B; mean absorbance in non-urolithiasis goats: 0.551±0.040, urolithiasis goats: 0.797±0.068, p=0.0144, mean concentration in non-urolithiasis goats: 5.762±0.563, urolithiasis goats: 9.228±0.954, p=0.0144). The Oxalometer also yielded similar results (Figure 3C and 3D: mean absorbance in non-urolithiasis goats: 0.050±0.008, urolithiasis goats: 0.126±0.018, p=0.0109; mean concentration in non-urolithiasis goats: 4.254 µM±0.772, 10.554µM±1.401, p=0.0028). Mann-Whitney t-test - *P<0.05, **P<0.005