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Research article

A simple and reliable refractometric method to determine the total solids concentration of the cervico-vaginal bovine mucus samples

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ABSTRACT

Cervico-vaginal mucus (CVM) is a viscoelastic substance continuously produced by secretory cells of the endocervix and the vagina of cows. Its physicochemical composition varies depending on the hormonal status of the estrous cycle. In veterinary medicine refractometry is a widely diffused technique to determine total solids (TS) content of biological samples, but there are not published data of CVM total solids from refractometric measures. Refractometric TS determination contributes to the qualitative constituents analysis of CVM, additionally it is an easier and more inexpensive technique than gravimetric TS determination. The main goal of the present paper was to validate a refractometric method to estimate TS concentration of the soluble fraction of CVM samples. Samples were collected from seventy-three Holando Argentino cows of Santa Fe province farms in Argentina. Cows were classified in three experimental groups: healthy, subclinical (SE) and clinical endometritis (CE) group. To achieve a solubilisation protocol for CVM samples, four Triton™ X-100 concentrations were tested. Refractive index (RI) and gravimetric total solid (gTS) concentration of solubilised samples were determined for the three experimental groups. A mathematical equation was determined with the experimental data from the healthy group, in order to obtain calculated total solid concentration (cTS) from refractivity (R) values. To validate the RI method for CVM samples, cTS concentrations were compared with gTS concentrations from endometritis group samples. Triton™ X-100 0.01% (V/V) improved CVM samples handling and did not change physicochemical parameters (gTS, Na^+ and K^+ concentration, and RI values). The linear regression equation obtained was: cTS (g/dL) = (R – 0.67)/16.2, $r^2 = 0.91$. Correlation between gTS and cTS concentration was: r = 0.97 for SE group and r = 0.97 for CE group. The homogenization protocol allowed the measurement of physicochemical parameters without altering their values. A high correlation coefficient between cTS and gTS postulates refractometry as an accurate method to determine TS concentration for solubilised CVM samples.

1. Introduction

The goal of reproduction management is to have cows become pregnant at a biologically optimal time and at an economically profitable interval after calving [1], because the reproductive efficiency has a major impact on economic success of any dairy production unit [2]. Poor fertility, low milk production and the direct cost of treatment result in significant loses to the dairy industry.

The postpartum bovine uterus is susceptible to bacterial contamination, viral infection as well as endometritis causing uterine disease [1, 3]. Cervico-vaginal mucus (CVM) can be easily collected and does not require special training therefore provides a more accessible resource to assess disease status. CVM represents a mixture of vaginal, cervical and uterine mucus [4], continuously produced by secretory cells of the endocervix and of the anterior vaginal epithelium. The mucus protects the bovine reproductive tract by maintaining the epithelial surfaces moist and lubricated [4, 5] and its quantity and composition changes depending on the hormonal status of the estrous cycle [6]. CVM is a hydrogel composed of 92–95% water, amino acids, lipids, carbohydrates, ions such as Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Cr^{2+}, Cd^{2+}, Hg^{2+}, proteins and enzymes [7, 8, 9]. Inorganic salts in mucus amount to 1%, of which the principal constituent is sodium chloride (NaCl) [8]. The NaCl

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is responsible, along with potassium ions, for the crystallization phenomenon, which is also known as the fern reaction [8]. The gel phase of the mucus is composed of mucin-type glycoproteins [4], and these probably constitute the main factor responsible for the rheological properties of mucus [5]. CVM constitutes the first "filter" for spermatozoa and takes part in sperm selection, transport, and probably prevent early acrosome reaction at uterine cervix region [5, 10].

In veterinary medicine, refractometry is a widely diffused technique to determine total solids (TS) content of biological samples. Commercial refractometers have a TS scale used to measure urine specific gravity, blood plasma total protein concentration, and serum water estimation [11]. Refractometry is a simple and inexpensive technique, and it is routinely used to determine solute concentration in various body cavity fluids such as peritoneal and pericardial [11]. Refractive Index (RI) is the change of the light refraction angle produced by the concentration of sample solutes versus the refraction of light in the air. The RI of the solution is directly proportional to the total solid concentration of the sample [11]. Since RI can approximate the total solid percentage in liquids, it is a commonly used tool to evaluate the protein concentration and total solid percentage in colostrum or serum of cows. Refractometry has not been used to determine the total solid percentage in uterine fluid [12]. For this reason, it is absolutely necessary to improve the experimental protocol to solubilise the mucus, so that, it is possible to use the liquefied CVM sample to determine the RI of the resulting solution.

The main goal of the present paper was to validate a refractometric method to estimate total solids concentration of the soluble fraction of CVM samples.

The validity of refractometric coefficients to determine total solids has not been fully tested for cervico-vaginal mucus samples from cows.

2. Materials and methods

2.1. Animals and geographical localization

All experiments described in the present manuscript were carried out with permission of the Ethical Committee from the Faculty of Veterinary Sciences of the National University of Litoral, Argentina (Protocol Nº 146/12). This study was performed concerning animal welfare and ethical standards, and it was carried out from July 2017 to December 2019. Cows used in this study belong to three different dairy farms, located in the southern area of Santa Fe province in Argentina.

Samples were obtained from seventy-three Holando Argentino cows of different ages in good body condition. Cows included in this study were between three to eight weeks postpartum. All animals were free of anatomical abnormalities of the reproductive tract, and were sampled only once.

CVM samples were collected by a veterinary physician, and scored as: Type 0 = clear mucus, Type 1 = mucus with flecks of pus (≤50% pus), Type 2 = mucopurulent discharge (>50% pus), and Type 3 = purulent discharge (entirely pus) [13] and/or bloody purulent, brownish-red in color, which may be associated with a fetid or putrid odor. There were three experimental groups of cows: 1-healthy group (control group) of thirty cows which had cervical mucus type 0, without pus or visible infection; and negative endometrial cytology; 2- subclinical endometritis (SE) group of nineteen cows with cervical mucus type zero and positive endometrial cytology, and 3- clinical endometritis (CE) group of twenty-four animals with purulent cervical mucus (Type 1, 2 and 3). Representative pictures of each group are shown in Figure 1.

2.2. Collection of cervico-vaginal mucus (CVM) and sample treatment

The vulva of the cows was washed with a wet towel, and then dried with paper towel. To reach the cervix an insemination gun was used, and cervical mucus samples were collected using a plastic cannula attached to a 60 mL syringe. Samples of cervical mucus were collected by gentle aspiration from the cervix, quickly cooled and transported to the laboratory, where they were aliquoted and stored at -20 °C for later use. At adequate time, CVM frozen samples were defrosted at 37 °C in a thermostated bath with stirring for 10 min and processed according to the experimental protocol.

2.3. Cytobrush technique

Transrectal palpation of the internal genital organs was performed to evaluate the reproductive tract of cows with cervical mucus type zero. The sampling instrument was composed of an endocervical brush (Medibrush XL, Medical Engineering Co, SA) cut in its handle to approximately 5 cm long and attached to the mandrel of a stainless steel insemination gun. The instrument was covered with a sanitary plastic sleeve for protection from vaginal contamination.

Once the sampling instruments had passed through the cervix, in the base of the larger horn, the brush was exposed and endometrial cytology samples were collected by rotating the cytobrush in a clockwise direction while in contact with the uterine mucosa. Next, the brush was retracted into the sheath and the insemination gun was removed from the uterus and vagina. The brush was rolled on a slide and samples were stored for later staining in the laboratory (Staining 15®, Biopur, Argentina). The cows were diagnosed as SE positive when polymorphonuclear neutrophils (PMN) ≥ 5% [14].

2.4. Experimental protocols

The analysis was performed using the soluble fraction of CVM samples from healthy cows (control group), based on the fact that there are insoluble substances that alter RI values. CVM samples were solubilised by adding a non-ionic detergent, Triton™ X-100. To validate the utility of the protocol, the first step was to determine the minimum detergent concentration that solubilises CVM samples without altering its physicochemical properties. The second step was to measure RI and gravimetric total solids (gTS) of CVM solubilised samples. Therefore, the third step was to set an equation in order to obtain calculated total solids (cTS) concentration, from RI measures. In the fourth step, the same parameters

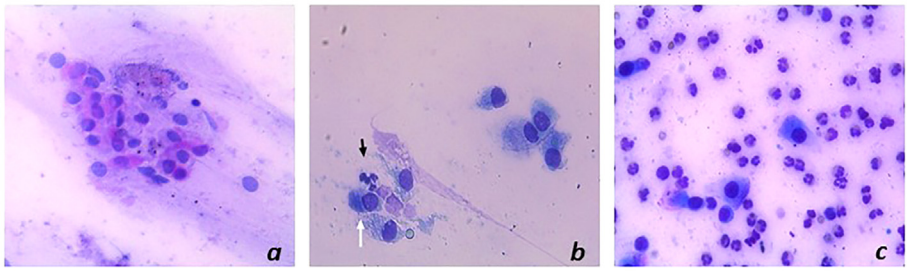

Figure 1. Representative pictures of cytology in control, SE and CE groups. Endometrial cytological evaluations. 400x. Staining 15®, Biopur, Argentina. a) Control group cytobrush slide shows normal endometrial cells b) SE group cytobrush slide shows endometrial cells (white arrow) and polymorphonuclear neutrophils (black arrow) c) CE group cytobrush slide shows great infiltration of PMN, cellular debris in the background and endometrial cells.

(RI, gTS and cTS) were determined for the clinical and subclinical endometritis groups. Finally, cTS concentrations were compared with gTS concentrations for endometritis groups, intended to validate the refractometric method.

2.4.1. Assessment of the minimum Triton™ X-100 concentration that homogenizes CVM samples and leaves no residues after complete desiccation

Triton™ X-100 is a common non-ionic surfactant and emulsifier which is often used in biochemical applications to solubilise proteins. It is considered a mild detergent, non-denaturing, and is reported in numerous references as a routinely added reagent. Four Triton™ X-100 solutions within the concentration range from 0.01 to 3 % (V/V) were tested to solubilise CVM samples. The effects of this addition on gTS concentration were determined using a horizontal forced air drying oven at 90 °C, until complete desiccation of each sample and weighted in an analytical balance. Each one of the defrosted CVM samples (n = 15) from control group was split in two, one half was added with a final concentration of 0.01% Triton™ X-100 (20μL 0.1% Triton™ + 180μL CVM samples) and in the other, the detergent was replaced with an equivalent volume of distilled water. All samples were homogenized with IKA-Ultra-Turrax™ T25 Basic, at minimum speed for 30 s, then they were centrifuged for 5 min at 12.000 *g* (Eppendorf AG™, MiniSpin™, Germany), and supernatants were used for physicochemical determinations. To evaluate if gTS content of the solubilised fraction of CVM samples truthfully represents TS content of raw samples, the Na^+ and K^+ concentration of both CVM samples, with and without surfactant were determined with a flame Photometer (Zentec ZF™ 2500, Argentina).

2.4.2. Effect of Triton™ X-100 addition on RI

All samples were homogenized as described in 2.4.1. RI was measured with a portable refractometer (ALLA FRANCE™, 95000-017, France), designed for a refractometric index measurement range between 1.3350 and 1.3600. All samples were measured according to Rodriguez [11].

To demonstrate that the results were measured without bias, a digital refractometer (ATAGO™ PAL-RI, catalogue N°3850, Japan), designed for a refractometric index measurement range between 1.3306 and 1.5284 was also used.

2.4.3. Match between gTS and refractivity values of CVM samples

From now on, all samples were homogenized as described in 2.4.1, defrosted samples were all added with a final Triton™ X-100 concentration of 0.01% V/V. Afterwards, RI and gTS of CVM solubilised samples were measured. The gTS values obtained were also used to calculate water content in each sample. For graphical reasons RI values were transformed into refractivity values using the following formula:

$$R = 10.000 \times (n_s - n_0)$$

R = refractivity
n_s = sample refraction index
n_0 = distilled water refraction index (blank reagent 0.01% Triton™ X-100 refraction index)

Linear regression plot between two variables: gTS (g/dL) against refractivity (R), obtaining the mathematical equation that relates them. This equation allowed the mathematical determination of cTS concentration measuring RI with a portable refractometer.

2.4.4. Determination of cTS for endometritis groups

Calculated total solids values were mathematically determined using the equation from section 2.4.3 and gTS concentrations were obtained using a horizontal forced air drying oven at 90 °C, until complete desiccation of each sample and weighted in an analytical balance. CVM samples of SE group (n = 19) and CE group (n = 24) were used to compare gTS with cTS values.

2.4.5. Statistical methods

Data of individual cows were exported to a spreadsheet file from the manually collected data. GraphPad® Prism5 software was used for the statistical analyses, descriptive statistics, correlation analysis and linear regression. Students T test was used to compare data between two groups. One-way ANOVA test was used to compare data between three groups. Results were presented as mean ± SD and considered statistically significant at $P < 0.05$.

3. Results

3.1. Effects of Triton™ X-100 on CVM samples

All Triton™ X-100 concentrations tested achieve mucus liquefaction, however, only 0.01% (V/V) Triton™ X-100 produced a negligible amount of foam, and improved CVM samples handling. In addition, the other three Triton™ X-100 concentrations tested left solid waste after complete desiccation and they produced a greater amount of foam (Table 1).

There were no statistical differences in gTS (P = 0.70), Na^+ (P = 0.24) and K^+ (P = 0.32) concentration values between CVM samples solubilised with 0.01% (V/V) Triton™ X-100 or distilled water (Table 2). High agreement (P = 0.19) between RI values of CVM samples measured with a digital refractometer and with a portable refractometer (Table 2).

3.2. Effect of Triton™ X-100 on RI

There were no statistical differences (P = 0.35) in RI values between samples processed with distilled water and samples added with 0.01% (V/V) Triton™ X-100. Descriptive statistical analysis results are shown in Table 2.

3.3. Match between gTS and refractivity values of CVM samples

The results of the descriptive statistical analysis from control group samples were: R = 33 ± 13, gTS 1.99 ± 0.77 (g/dL) and the water content 97.8 ± 0.9 (Table 3). Figure 2 shows a linear regression between gTS and refractivity (R) with a slope of 16.2 with a coefficient of regression (r^2) of 0.91. The equation for the mathematical determination of cTS was:

$$cTS\ (g/dL) = (R–0.67)/16.2 \qquad \text{Eq. (1)}$$

3.4. Comparison between gTS and cTS for endometritis groups

All cTS values were calculated using Eq. (1) obtained in section 3.3. and there were no differences between cTS and gTS from SE (P = 0.07) and CE (P = 0.18) group. Figures 3 and 4 show a high positive correlation between cTS and gTS values r = 0.97 for CE group and r = 0.97 for SE group.

Table 1. Selection of concentration Triton™ X-100.

Triton™ X-100 concentrations (V/V)	0.01%	0.5%	1%	3.3%
Liquefaction	+++	+++	+++	+++
Foam formation	+	+++	+++	+++
Solid waste (g/dL)	ND	0.71 ± 0.30	3.8 ± 0.30	4.02 ± 0.03

Control samples randomly selected were analyzed with different detergent concentrations (n = 8). Rank: - nothing, + little, ++ medium, +++ high. ND: non detectable.

Table 2. Comparison of Triton™ X-100 0.01% (V/V) effect in CVM samples.

	TS (g/dL)	Na^+ (meq/L)	K^+ (meq/L)	RI Portable refractometer	RI digital refractometer
CVM and distilled water	1.60 ± 0.90	113 ± 17	13 ± 2	1.3360 ± 0.0013	1.3350 ± 0.0009
CVM and Triton™ X-100 0.01% (v/v)	1.62 ± 0.97	103 ± 25	12 ± 4	1.3360 ± 0.0016	1.3360 ± 0.0008

TS (g/dL), Na^+ (meq/L), K^+ (meq/L), and refractive index (RI) values of CVM samples from control group cows treated with water or Triton™ X-100 0.01% (V/V). Values are expressed as means ± SD of duplicate samples (n = 15).

Table 3. Descriptive statistical analysis from control and endometritis groups.

	RI	R	gTS (g/dL)	cTS (g/dL)	Water content (%)
Control Group	1.3360 ± 0.0013	33 ± 13	1.99 ± 0.77	1.99 ± 0.81	97.8 ± 0.9
SE Group	1.3370 ± 0.0016	35 ± 15	2.24 ± 1.01	2.14 ± 0.92	97.8 ± 0.9
CE Group	1.3380 ± 0.0024	46 ± 24	2.70 ± 1.47	2.80 ± 1.46	97.2 ± 1.4

Refractive index (RI), refractivity (R), gravimetric total solids (gTS) (g/dL), calculated total solids (cTS) (g/dL), and water content of control (n = 30), SE (n = 19) and CE (n = 24) groups. Values are expressed as means ± SD of duplicate samples.

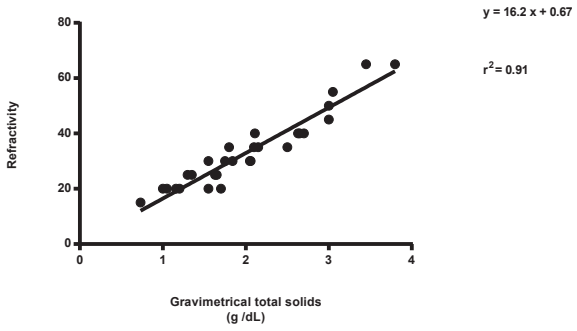

Figure 2. Linear regression (Eq. (1)) between gravimetric total solids (gTS) (g/dL) and refractivity values of control group CVM samples (n = 30).

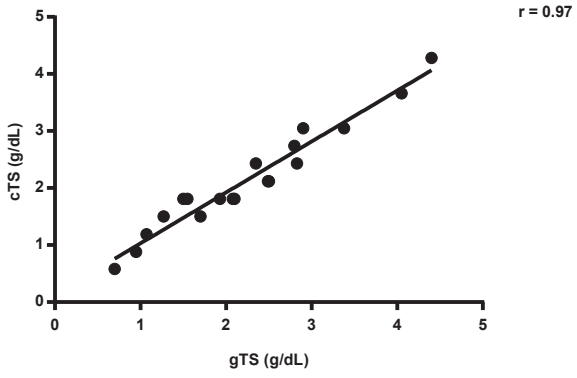

Figure 3. Correlation plot between gravimetric total solids (gTS) (g/dL) and calculated total solids (cTS) (g/dL) values of subclinical endometritis group CVM samples (n = 19).

A strong positive linear regression was obtained between TS concentration and refractivity (R) values of CVM solubilised samples from the healthy group of cows (n = 30), then the mathematical equation from section 2.4.3 was used to determine TS concentration for the other two endometritis groups. Furthermore, a high positive correlation was also found between cTS and gTS values of the two endometritis groups.

3.4.1. Statistical analysis to compare refractivity, gTS and water content among different CVM groups

Descriptive statistical analysis results of endometritis groups are shown in Table 3. There were no statistical differences in gTS (P = 0.07) and water content (P = 0.14) values between control, SE and CE groups. However, there were statistical differences in refractivity values (P = 0.02) among experimental groups. Refractivity values were statistically different only between control group and CE group (P = 0.01).

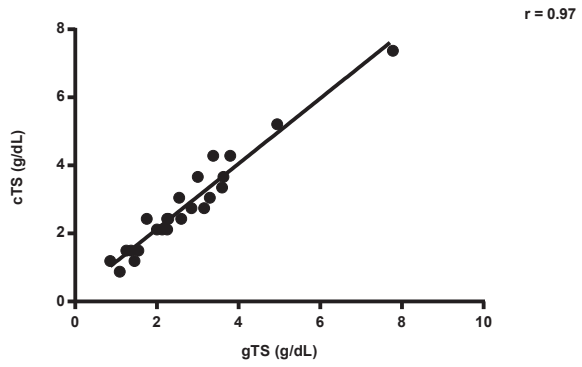

Figure 4. Correlation plot between gravimetric total solids (gTS) (g/dL) and calculated total solids (cTS) (g/dL) values of clinical endometritis group CVM samples (n = 24).

4. Discussion

Triton™ X-100 addition to CVM samples at a final concentration of 0.01% (V/V) attain mucus liquefaction without leaving residues after complete desiccation and facilitated mucus manipulation for further physicochemical determinations. However, the other three Triton™ X-100 concentrations tested left residues and produced a greater amount of foam, which worsened samples handling. Furthermore, there were no statistically significant differences in the physicochemical properties tested (gravimetric total solid concentration, refraction index, refractivity, Na^+ and K^+ concentration) between Triton™ X-100 0.01% (V/V) and distilled water treatment.

Due to the physical properties of mucus, processing of CVM with reducing agents is routinely recommended before the analysis of soluble-phase biomarkers [3]. N-acetyl-L-cysteine (NAC) and Dithiothreitol (DTT) are commonly used to homogenize mucus by reducing the disulfide bonds of mucins [3]. However, many immune biomarkers also have disulfide bonds and their detection is likely to be compromised by use of reducing agents [3]. Alternatively, the non-ionic detergent Triton X-100 is one of the most routinely used agents to solubilise biomembranes [15]. Non-ionic detergents are generally considered to be mild and relatively non-denaturing, as they break lipid–lipid and lipid–protein interactions rather than protein–protein interactions [16]. This allows many membrane proteins to be solubilised in non-ionic detergents without affecting the protein's structural features, such that it can be isolated in its biologically active form [16].

Refractivity values of CVM solubilised samples from healthy animals were within a range from 15 to 65 and the mean water content was almost 98%, similar to those published by Tsiligianni [7]. There were no statistically significant differences between RI values of CVM samples measured with a digital refractometer and a portable refractometer.

Variations among CVM sample refractivity values from cows of the same experimental group may be related to physicochemical changes of the mucus according to the stage of estrous cycle. The variations in the levels of estrogens and progesterone during the cycle have a marked effect on composition, physicochemical and structural properties, and rheological attributes of the mucus [5]. CVM water content varies along the cycle, increasing at estrus, due mainly to the rise in estradiol levels observed in this stage. Also, CVM types are differentially expressed during the stages of the estrous cycle, a change also related to fluctuations in sex steroid hormones [5]. For instance, during the periovulatory period (estrogens increase) mucus secretion raises, becoming less viscous and more hydrated [5]. Also, in this period, the mucus structure would facilitate the movement of normal spermatozoa [5, 17]. Additionally, inhibit the ascent of gametes with morphological alterations, acting as a selective filter [5, 18]. In the luteal phase, the amount and hydration of the secreted mucus decreases while its viscosity increases, preventing spermatozoa migration [5]. For this reason, the refractivity values of CVM samples could be used to determine fluctuations in total solids concentration throughout the estrous cycle.

Clinical and subclinical endometritis in dairy cows continue to be a major financial and animal welfare impediment to the global dairy sector [12]. Clinical diseases with apparent signs are more easily diagnosed and treated [12]. However, diagnosis of subclinical uterine disease can be more difficult [12]. SE can be diagnosed in different ways, including endometrial biopsy, endometrial cytology via cytobrush, and endometrial cytology via low-volume lavage [12]. SE is often under-diagnosed by practitioners and producers because a simple, rapid diagnostic test is not available [12]. CVM samples could be used as a more accessible resource for cow-side tests to assess uterine health. Refractivity values in the present study were significantly different between control and CE group, whereas there were no statistical differences between control and SE group. Additionally, there was statistical dispersion of refractivity values among CVM samples in each experimental group. As mentioned before, this variation could be due to the fact that cows were sampled in a period of five weeks postpartum and the animals were in different stages of the estrous cycle. Therefore, more research should be done in order to validate refractivity as a cow-side technique for SE diagnosis.

There are many advantages in using a hand refractometer to determine TS of CVM samples, such us, it is a simple and inexpensive technique, it requires a very small sample volume and is widespread in veterinary clinic. The present study also contributes to the analysis of the qualitative constituents of cervico-vaginal mucus, since there are RI and R values for other biological fluids but there were not published values for CVM samples.

5. Conclusions

A linear regression coefficient of 0.91 represents a very strong relationship between refractivity and gravimetric total solids, validating the mathematical formula (Eq. (1)) proposed to determine TS concentration from Refractivity measures. Moreover, gTS and cTS values of CVM samples from endometritis groups showed a strong relationship demonstrating that the method is accurate.

Declarations

Author contribution statement

Caren L. Savia, Juliana S. Osorio: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Joaquín V. Rodríguez, Edgardo E. Guibert, Agustín Rinaudo: Conceived and designed the experiment; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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