Novel bioactive caries-detecting dye solution: Cytotoxicity, antimicrobial activity, scanning electron microscope, and stereomicroscopic analysis in diagnosis of dental caries

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Abstract

Aim: The aim was (1) to study the cytotoxicity of novel Bioactive Caries-detecting Dye solution (BCD) and its antimicrobial activity against Streptococcus mutans, Lactobacillus acidophilus, Actinomyces naeslundii, and Candida albicans and (2) comparative assessment of BCD and Carie-Care for efficient removal of caries (stereomicroscope) and dentin tubule occlusion (scanning electron microscope [SEM]).

Materials and Methods: For BCD cytotoxic study (direct contact method), colorimetric MTT assay, and cell line study (L929 mouse fibroblast NCTC clone 929 strain L) was performed. Xenetix 350, chitosan, nanohydroxyapatite (nHA), BCD, and Carie-Care solutions were subjected to the antimicrobial activity through blood agar well diffusion method, and the minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined. On 20 extracted human carious teeth a comparative pilot study was done for BCD (Group A, n = 10) and Carie-Care (Group B, n = 10), and evaluated visually and radiographically. After mechanical excavation of caries with a spoon excavator, teeth sectioned longitudinally and stereomicroscopically were evaluated (8x–40x) by two observers. The percentage of dentinal tubule occlusion was evaluated with SEM for both solutions. Statistical kappa analysis of agreement was 0.7–0.8 (P < 0.01). Mann–Whitney test ranks and Wilcoxon signed-rank test (P = 0.01) were applied.

Results: Cytotoxicity test revealed BCD to be nontoxic and biocompatible. Antimicrobial tests (zone of inhibition) showed BCD > chitosan > chlorhexidine > Carie-Care > Xenetix 350 > nHA. MIC and MBC values suggested chlorhexidine > BCD > Carie-Care. Stereomicroscopic analysis showed effective mechanical removal of caries in BCD without residual dye in the dentinal tubules as compared to Carie-Care. Dentinal tubule occlusion (SEM analysis) was 80%–85% for BCD and 10% for Carie-Care.

Conclusions: Profound synergistic effect for BCD was observed with advantage of radiographic assessment.

Keywords: Antimicrobial activity; bioactive caries-detecting dye; Carie-Care; caries diagnosis; cytotoxicity; scanning electron microscope, stereomicroscope

INTRODUCTION

Decades have passed to eradicate the most common, widely spread infectious microbial disease “dental caries.”

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How to cite this article: Shashirekha G, Jena A, Mohanty N, Kamilla SK. Novel bioactive caries-detecting dye solution: Cytotoxicity, antimicrobial activity, scanning electron microscope, and stereomicroscopic analysis in diagnosis of dental caries. J Conserv Dent 2020;23:79-85.
Epidemiologically complete eradication is not possible as it involves various hidden factors. Revelation regarding critical factors involved in the process of caries formation shows that microorganisms have a significant role. Advanced techniques have been invented and adapted to diagnose and prevent dental caries.

Along with the traditional diagnostic methods, various techniques have evolved to identify, disinfect, and manage the demineralized enamel/dentin and prevent secondary caries formation. Extensive studies have been carried out with conventional techniques and materials (visual, radiographic method and caries-detecting dyes) and advanced methods (QLF, DIAGNOdent, Calcivis, etc.). Caries dyes and chemomechanical caries removal (CMCR) methods are considered minimally invasive for the identification and management of dental caries[1-4] but are time-consuming with compromised clinical outcomes. Therefore, to overcome these clinical challenges, our research team formulated a composition: bioactive caries-detecting dye solution (BCD) for the identification of dental caries clinically and radiographically.

Thus, the present study investigates: (1) the cytotoxicity of BCD and antimicrobial activity of BCD and Carie-Care (Innovation-Hub5, Bengaluru, Karnataka, India) against Streptococcus mutans, Lactobacillus acidophilus, Actinomyces naeslundii, and Candida albicans and (2) the comparison of BCD and Carie-Care for efficient removal of caries (stereomicroscope) and dentine tubule occlusion (scanning electron microscope [SEM] analysis).

MATERIALS AND METHODS

All experiments were done after approval from Siksha ‘O’ Anusandhan (Deemed to be) University/Institutional Ethical Committee, Odisha, India (number: DMR/IMS. SH/SA/180168)(IP India Patent application no: 201831039005).

Preparation of BCD
Iobitridol (Xenetix 350, Guerbet, Aulnay-Sous-Bois, France), 3% chitosan (i-CHESS, Mumbai, Maharashtra, India), 15% nHA (NanoXIM Care, Fluidnova, Maia, Portugal), and laccasia acid were used in this study. A homogeneous solution was obtained using an electromagnetic stirrer, and the pH was determined with digital pH meter (Eutech pH 700, Thermo Fisher Scientific, USA) as 6.5–7.

Cytotoxicity and cell viability assay for BCD
In vitro cytotoxicity by the direct contact method (sample preparation followed as per ISO standards), colorimetric MTT assay, and cell line (L929 mouse fibroblast NCTC clone 929 strain L) was used for the study.[5-7]

Preparation of test materials
Test material (BCD) was autoclaved and diluted in culture medium to the final concentration of 5% (v/v). Test procedure: L929 mammalian fibroblast cells were grown to subconfluence (approximately 80%) in a culture plate. After verifying subconfluence, the culture medium was removed and discarded. 100µl of the test material was added into test wells in triplicate. The culture medium served as a negative control, and dilution of dimethyl sulfoxide (DMSO) served as a positive control. Plates were incubated at 37°C with 5% CO2 and >90% humidity for 24 h and subsequently examined under a phase-contrast microscope for assessing changes in general morphology, vacuolization, detachment, cell lysis, and membrane integrity. After microscopic examination, test wells were rinsed with culture medium to remove traces of test material. Fresh culture medium and MTT solution were added to each well, and the plate was swirled to mix the dye and incubated (Mid40, Thermo Scientific, USA) in dark for 3 h at 37°C. After 3 h, formazan crystals formed were dissolved in solvent and absorbance was detected at 570 nm (reference 690 nm) on a plate reader.

Qualitative morphological grading of cytotoxicity by microscopic observation scored from 0 to 4:
• 0 = No reactivity; discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth
• 1 = Slight reactivity; not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable
• 2 = Mild reactivity; not more than 50% of the cells are round, devoid of intracytoplasmic granules, no extensive cell lysis, not more than 50% growth inhibition observable
• 3 = Moderate reactivity; not more than 70% of the cell layers contain rounded cells or lysed; cell layers not destroyed, but more than 50% growth inhibition observable
• 4 = Severe reactivity; almost or destruction of the cell layers.

Quantitative measurements of cytotoxic effects by MTT assay by applying Viability%=$100 \times \frac{OD_{570c}}{OD_{570t}}$
OD$_{570c}$ is the mean value of the measured optical density of the test material after blank subtraction. OD$_{570t}$ is the mean value of measured optical density of the negative control after blank subtraction.

Antimicrobial assay
S. mutans microbial type culture collection (MTCC) 497, Lactobacillus salivarius MTCC 12829, A. naeslundii ATCC® 12102®, and C. albicans MTCC 183 were obtained from MTCC, Chandigarh, India. All microorganisms were reactivated in brain–heart infusion broth (HiMedia, Mumbai, India) at 37°C for 24 h in aerophila. 2% chlorhexidine digluconate (FGM, Joinville, SC, Brazil) and 10% DMSO served as a positive and negative control, respectively. For the growth
of *C. albicans*, Sabouraud dextrose agar (SDA) was used as a selective medium. Nystatin discs (HiMedia, Mumbai, India) were used as a control disc.

The *in vitro* antimicrobial activities of the five different test solutions, i.e., Iobitridol, 3% chitosan, nHA, Carie-Care, and BCD were determined using two methods: (a) agar well diffusion method and (b) microdilution method.

**Agar well diffusion method**

This procedure evaluated the antibacterial activity. 100 µl Mueller-Hinton agar for *S. mutans*, *L. salivarius*, and *A. naeslundii* and SDA for *C. albicans* plates spread with an L-shape spreader with fully grown broth culture of respective bacteria were used. Further, three equidistant 0.5 mm wells were bored on each plate with the help of a sterile cork borer. 50 µl of three different concentrations (10 mg/ml, 30 mg/ml, and 50 mg/ml) was added to the wells using a micropipette and allowed to diffuse at room temperature in the laminar airflow for 30 min. Control experiments comprised inoculums with 10% DMSO only. Plates were then incubated at respective temperature, i.e., 30°C and 37°C (18–24 h) for bacterial pathogens. The diameter of the zone of inhibitions (in millimeters) was measured after the incubation period. Experiments were performed in duplicate, and the readings were noted. 2% chlorhexidine digluconate was used as a positive control.

**Microdilution method**

A comparison of different bacterial species was difficult since they had different optical densities, growth timing, and requirements. Furthermore, at a time, the number of bacteria of different bacterial species in their respective medium is different. To overcome this problem, a uniform number of bacteria were used. The optimum concentration of 5 × 10³ CFU/ml was implemented for this test to compare different strains and species.

**Preparation of test culture**

Under aseptic conditions, a single colony was inoculated into a 5 ml test tube containing its respective broth, then capped, and placed in the incubator at its respective temperature for its respective time at 200 rpm. After incubation, 100 µl of culture was transferred into a 10 ml test tube containing its broth. Again, it was placed in the incubator until its optical density, OD600 obtained at 0.5. When the absorbance was 0.5, serial dilution was done. Here, 10⁻³ dilutions were taken. Similarly, all the strains were prepared for this assay.

**Preparation of plates**

Sterile 96-well plates were coated with resazurin, media, drug, and bacteria under aseptic conditions inside the laminar airflow. A stock solution of 50 mg per ml 10% DMSO for test solutions was prepared. Four different lower concentrations were prepared (10 mg/ml, 20 mg/ml, 30 mg/ml, and 40 mg/ml). Wells were filled up with 50 µl of broth media, 50 µl test solutions in different concentrations, 10 µl resazurin indicator solution, and 10 µl of bacterial suspension (5 × 10³ CFU/ml). Two blank wells were made, first with no drug and second with no bacterial culture. Each plate was wrapped loosely with aluminum foil to ensure that bacteria did not get dehydrated. The plates were prepared in duplicates and kept in incubator at 37°C for 18–24 h, and color changes were assessed visually. The color change from purple to pink or colorless was recorded as the presence of bacteria. The minimum inhibitory concentration (MIC) value of test solutions is the lowest concentration at which color does not change to pink and remains blue. 2% chlorhexidine digluconate, a broad-spectrum antibiotic, was taken as standard. MIC of the control group and for the four bacterial strains was done separately. 1 mg/ml stock solution and further different concentrations were prepared. For evaluation of minimal bactericidal concentration (MBC) values, 10 µl from each plate well that exhibited no growth was taken and incubated at 37°C for 18–24 h. MBC was noted as the lowest concentration that revealed no visible bacterial growth after subculturing.[8]

**Stereomicroscopy and scanning electron microscope evaluation**

Pilot study was done with twenty extracted human carious teeth (occlusal and proximal caries, n = 10 each) of unknown history as per University guidelines. Teeth were disinfected with 0.2% thymol (DWD Pharmaceuticals Ltd, Mumbai, Maharashtra, India), and stored in artificial saliva (Wet Mouth, ICPA Health Products Ltd, Mumbai, Maharashtra, India) until used. The teeth were randomly divided (www.random.org) into two groups.

Group A (n = 10) – BCD – occlusal carious teeth (A1), n = 5, and proximal carious teeth (A2), n = 5. Group B (n = 10) – Carie-Care – occlusal carious teeth (B1), n = 5, and proximal carious teeth (B2), n = 5.

Teeth were mounted with modeling wax till the cementoenamel junction level. BCD was applied on carious teeth with a micro-brush and scrubbed for 20 seconds under 16x magnification. In Group B, Carie-Care was applied for 30 seconds according to manufacturer instructions. When the gel became cloudy, it was removed by scraping with spoon excavator and gel was again applied until it was no longer cloudy. For both the groups, radiovisiography (RVG) (FONA CDRelite) was taken to view the extension of the dye which indicated the extension of the carious lesion and scored. For Group A, softened carious lesion was removed with a spoon excavator after 60 s. Three teeth from each subgroup (A1, A2, B1,
and B2) were sectioned with a low-speed, water-cooled diamond disc saw in a mesiodistal plane. Both sides of each section were viewed at 8x to 40x under transmitted light using stereo-microscope. (Motic GM-168, Hong Kong) [Figures 1 and 2a, b, d-f]. Photographs were taken at each step (preoperative photographs, after the application of dye, and after removal of caries). For both the groups, two observers evaluated the efficacy of dye ingress and removal of caries visually and radiographically. Scoring was done according to the modified American Dental Association Caries Classification as R0 (E0) – no radiolucent/radio-opaque, RA-1-3 (E1, E2, and D1) – radiolucent/radio-opaque may extend to the dentinal enamel junction or outer-third of the dentine, RB4 (D2) – radiolucent/radio-opaque into the middle one-third of the dentin, and RC5 (D3) – radiolucent/radio-opaque extends into the inner one-third of the dentin. Since Group B was radiolucent on RVG, scoring was “0.”

Visual scoring under stereomicroscope was classified as 0 – sound tooth structure, 1 – brown lesion in enamel, 2 – brown lesion in dentin, and 3 – brown lesion in enamel and dentin. The remaining four teeth from each group were restored with intermediate restorative material (Dentsply, USA), and SEM analysis was done to know the percentage of occluded dentinal tubules. The samples were thoroughly washed in distilled water, dried, and sputter-coated (Q150R ES, GS Quorum Technologies Limited, England) with a thin layer of gold, and photomicrographs were taken using SEM (Merlin Compact, Carl Zeiss, Germany) at ×2000 magnification and 5 kV.

RESULTS

Statistical analysis has been conducted using IBM SPSS statistics 24.0 SPSS South Asia (P) Ltd, Bengaluru, Karnataka, India, www.spss.co.in). Table 1 shows the cytotoxicity values of BCD; score 0 indicates no reactivity, discrete intracytoplasmic granules, no cell lysis, and no reduction of cell growth. 5% BCD had 80.35% cell viability. The

| Samples                        | Quantitative measurements (MTT assay) | Viability percentage (%) |
|-------------------------------|--------------------------------------|--------------------------|
| 5% BCD                        | 0                                    | 80.35                    |
| Negative control              | 0                                    | 100                      |
| Positive control DMSO 30%     | 4                                    | 4.32                     |

Table 1: Cytotoxicity assessment of BCD

BCD: Bioactive Caries-detecting Dye solution, DMSO: Dimethyl sulfoxide
characteristic features of microorganisms are described in Table 2. BCD had a higher zone of inhibition for four microorganisms as compared to other test solutions [Table 3]. MIC and MBC values showed that BCD has greater bacteriostatic and bactericidal properties as compared to Carie-Care but insignificant to the chlorhexidine [Table 4]. Stereomicroscopic analysis was subjected to statistical kappa analysis of agreement 0.7–0.8 ($P < 0.01$). The values of observer 1 were subjected to Mann–Whitney test ranks [Table 5] and Wilcoxon signed-rank test [Table 6] at $P = 0.01$. Visual examination for occlusal caries removal of Group A was significant compared to Group B ($P < 0.05$), but statistically insignificant for proximal caries removal between the groups. SEM analysis was calculated by dividing the total number of occluded tubules by the total number of tubules in the photomicrograph and then multiplied by 100 to obtain the percentage of occluded tubules for each photomicrograph. 80-85% of dentinal tubule occlusions were seen for the BCD group [Figure 3a-d], whereas 90% of open tubules were seen in the Carie-Care group [Figure 3e and f].

**Table 2: Illustrations of microorganisms**

| Microorganisms | Characteristic features |
|----------------|------------------------|
| *Streptococcus mutans* | Gram-positive facultative anaerobe. Grown in anaerobic condition with low percentage of oxygen with 5%-10% carbon dioxide. Colonies are either regular and smooth or irregular, hard, and sticky |
| *Lactobacillus salivarius* | Gram-positive nonsporulating bacilli. Grown in anaerobic or microaerophilic conditions. Colonies are smooth, round, raised white or gray, transparent or nontransparent |
| *Actinomyces naeslundii* | Gram-positive irregular bacillus, grown in an anaerobic environment without carbon dioxide. Mature colonies are convex or flat, rough, or smooth without center sag. No hemolytic reaction on blood agar |
| *Candida albicans* | Gram-positive large and spherical fungus, it is an aerobic microorganism grown best in temperature from 30°C -37°C, with 24-48 h. They grow well in Sabouraud agar media and form milk white, smooth surface, and softer yeast-like colony |

**Table 3: Zone of inhibition (in millimeters) of different solutions against microorganisms**

| Microorganism | Chlorhexidine (control group) | Xenetix 350 | Chitosan | nHA | Carie-Care | BCD |
|---------------|-------------------------------|-------------|----------|-----|------------|-----|
| *Streptococcus mutans* (MTCC No: 497) | 2 | 0.8 | 1.2 | 0 | 1.0 | 2.1 |
| *Lactobacillus salivarius* (MTCC No: 12829) | 1.6 | 1.5 | 0 | 0.9 | 1.9 |
| *Actinomyces naeslundii* ATCC® 12102™ | 1.8 | 0.5 | 1.6 | 0 | 3 | 4 |
| *Candida albicans* (MTCC No: 183) | 1.8 | 0.6 | 1.7 | 0 | 0.7 | 2 |

MTCC: Microbial type culture collection, BCD: Bioactive Caries-detecting Dye solution, nHA: Nanohydroxyapatite

**Table 4: Minimum inhibitory concentration and minimal bactericidal concentration (in µg/ml) of BCD and Carie-Care against all microorganisms**

| Microorganism | *Streptococcus mutans* (MTCC No: 497) | *Lactobacillus salivarius* (MTCC No: 12829) | *Actinomyces naeslundii* ATCC® 12102™ | *Candida albicans* (MTCC No: 183) |
|---------------|-------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
| MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| Chlorhexidine (control) | 100 | 125 | 100 | 150 | 100 | 125 | 100 | 175 |
| Carie-Care | 150 | 200 | 125 | 225 | 100 | 175 | 125 | 200 |
| BCD | 125 | 150 | 100 | 150 | 100 | 125 | 100 | 150 |

MIC: Minimum inhibitory concentration, MBC: Minimal bactericidal concentration, MTCC: Microbial type culture collection, BCD: Bioactive Caries-detecting Dye solution

**DISCUSSION**

In the current study, cytotoxicity test revealed BCD as a biocompatible material; therefore, on contact with dentin, pulpal tissue, and oral epithelium, no cytolytic and hypersensitivity reactions are expected. Cell viability of 5% BCD is 80.35%, and with a pH of 6.5–7, there is a minimum inflammatory response on pulpal and oral epithelium tissue. Solution with <70% viability and high alkalinity leads to denaturation of adjacent cells and proteins on initial contact. [10] The nonionic radio pacifier along with biomaterials lacks adverse cellular reaction
and shows the trend of increasing bone formation.\textsuperscript{[11]} nHA/glycolchitosan biocomposites have adequate cell viability response and non-cytotoxic behavior to a human osteoblast-like model cell line (SAOS) and embryonic cell lines (HEK293T).\textsuperscript{[12,13]} BCD containing chitosan biopolymer in combination with nHA produces an eco-friendly chemical colloidal process in water-based nonionic lobitrildol solution.

For antimicrobial tests, BCD showed significantly increased zone of inhibition against \textit{S. mutans}, \textit{L. salivarius}, \textit{A. naeslundii}, and \textit{C. albicans}, followed by chlorhexidine > chitosan > Carie-Care > Xenetix 350 > nHA [Table 4]. Based on MIC and MBC values, BCD showed bacteriostatic and bactericidal effects against the prime bacteria causing caries. nHA had no antibacterial effect, but chitosan showed a remarked zone of inhibition for all four microorganisms. Chitosan has good antibacterial, antifungal activity, chelation ability, and regenerative effect on connective tissues, accelerates the formation of osteoblast, and possesses absorptive capacity and ability to scavenge oxygen free radicles as it is polycationic and composed of 2-amino-2-desoxy-D-glycopyranose interconnected by glycosic bonds-1,4 in variable proportions.\textsuperscript{[14,15]} It has high reactive amino (−NH2) and hydroxyl groups (OH), and the degree of cell attachment is high, with an increase in the percentage of deacetylation of chitosan.\textsuperscript{[16,17]} For Xenetix 350, extensive clinical research has been done for its enhanced antibacterial property, safety, and efficacy.\textsuperscript{[18-20]}

Therefore, BCD with chitosan, nHA, and Xenetix 350 as active ingredients showed significantly higher zone of inhibition, as compared to Carie-Care which consists of papaya extract (endoprotein), chloramines, and dye.\textsuperscript{[21,22]} Stereomicroscopic images of cross-sectioned teeth showed the absence of dye penetration into the sound dentin for both the groups [Figures 1f and 2f]. Low molecular weight and reduced surface tension with high diffusion property may lead to deeper ingress of the dye into sound dentin.\textsuperscript{[23]} Therefore, both the groups can be considered as having high molecular weight.

BCD solution showed a synergetic effect in the identification of dental caries clinically and radiographically [Figure 1c], effective removal of demineralized dental tissues on a single application, potential reduction of dentin hypersensitivity (DH) and prevented secondary caries formation. The reason being chitosan is considered natural cationic adhesive that becomes viscous on being neutralized with acids. Various forms of chitosan-based delivery medium for amelogenin have been employed with the aim of rejuvenating the aligned crystal structure.\textsuperscript{[24,25]} nHA is an inert, favorable bioactive component, hydrophilic in nature, high wettability, and forms a thin and strong bond on the enamel layer of the tooth structure.\textsuperscript{[13]} Chitosan and nHA together create a homogeneous compound with high strength and adhesion properties,\textsuperscript{[25,26]} and their dual combination in BCD imparts protective effect against secondary caries and has corresponding antibacterial properties with influence on the reduction of DH.\textsuperscript{[27,28]}

Carie-Care extension cannot be evaluated radiographically [Figure 2c]. At present, no CMCR is radiopaque in nature; therefore, BCD will enhance the visibility of dental caries extension in radiographs.

**CONCLUSIONS**

The microbiota diversity responsible for dental caries can be managed considerably with BCD. Deliberate application of BCD can regularly monitor the occurrence and decrease the incidence of dental caries at any age group. Long-term clinical trials will be carried in the future.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

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**Table 5: Radiographic evaluation of BCD and Carie-Care**

| Radiographic evaluation | Groups | Mean rank | Mann-Whitney U | Significant |
|-------------------------|--------|-----------|----------------|-------------|
| Occlusal A1, B1         | Group A | 8.00      | 0.000          | 0.005       |
|                         | Group B | 3.00      |                |             |
| Proximal A2, B2         | Group A | 8.00      | 0.000          | 0.005       |
|                         | Group B | 3.00      |                |             |

**Table 6: Stereomicroscopic evaluation of BCD and Carie-Care**

| Groups\(^{a}\) | \(n\) | Mean rank | Sum of ranks | Mann-Whitney U | Wilcoxon W | \(Z\) | Asymptotic significant (two-tailed) | Exact significant (\(Z^2\) (one-tailed significant)) |
|--------------|-----|-----------|--------------|----------------|-------------|------|----------------------------------|----------------------------------|
| Visual       |     |           |              |                |             |      |                                  |                                  |
| Occlusal     |     |           |              |                |             |      |                                  |                                  |
| Dimension 1  |     |           |              |                |             |      |                                  |                                  |
| A            | 5   | 3.50      | 17.50        |                |             | -2.362 | 0.018                            | 0.032\(^{a}\)                    |
| B            | 5   | 7.50      | 37.50        | 2.50           | 17.50       |       |                                  |                                  |
| Total        | 10  |           |              |                |             |       |                                  |                                  |
| Visual proximal |     |           |              |                |             |      |                                  |                                  |
| Dimension 1  |     |           |              |                |             |      |                                  |                                  |
| A            | 5   | 4.40      | 22.00        |                |             | -1.315 | 0.189                            | 0.310\(^{a}\)                    |
| B            | 5   | 6.60      | 33.00        | 7.00           | 22.00       |       |                                  |                                  |
| Total        | 10  |           |              |                |             |       |                                  |                                  |

\(^{a}\)Not corrected for ties, \(^{a}\)Grouping variable: Groups
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