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RESEARCH ARTICLE

Developing affordable and accessible pro-angiogenic wound dressings; incorporation of 2 deoxy D-ribose (2dDR) into cotton fibres and wax-coated cotton fibres

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Abstract

The absorption capacity of cotton dressings is a critical factor in their widespread use where they help absorb wound exudate. Cotton wax dressings, in contrast, are used for wounds where care is taken to avoid adhesion of dressings to sensitive wounds such as burn injuries. Accordingly, we explored the loading of 2-deoxy-D-ribose (2dDR), a small sugar, which stimulates angiogenesis and wound healing in normal and diabetic rats, into both types of dressings and measured the release of it over several days. The results showed that approximately 90% of 2dDR was released between 3 and 5 days when loaded into cotton dressings. For wax-coated cotton dressings, several methods of loading of 2dDR were explored. A strategy similar to the commercial wax coating methodology was found the best protocol which provided a sustained release over 5 days.

Cytotoxicity analysis of 2dDR loaded cotton dressing showed that the dressing stimulated metabolic activity of fibroblasts over 7 days confirming the non-toxic nature of this sugar-loaded dressings. The results of the chick chorioallantoic membrane (CAM) assay demonstrated a strong angiogenic response to both 2dDR loaded cotton dressing and to 2dDR loaded cotton wax dressings. Both dressings were found to increase the number of newly formed blood vessels significantly when observed macroscopically and histologically.

We conclude this study offers a simple approach to developing affordable wound dressings as both have the potential to be evaluated as pro-active dressings to stimulate wound healing in wounds where management of exudate or prevention of adherence to the wounds are clinical requirements.

KEYWORDS

2-deoxy-D-ribose (2dDR), angiogenesis, chick chorioallantoic membrane (CAM) assay, cotton dressings, wound healing

1 | INTRODUCTION

In the development of wound dressings, the priorities are to provide a safe barrier layer to ensure wound cover and to create an environment which is conducive to wound healing. Over the years, these have been achieved with the use of a range of materials.

Wound dressings can be described as simple or advanced, and there are many excellent reviews of the strategies for developing these such as a recent review from Öhnstedt et al. (Öhnstedt, Lofton Tomenius, Vågesjö, & Phillipson, 2019). The natural process of wound healing can be delayed or arrested where there is a poor blood supply, bacterial or fungal infection (particularly bacterial biofilms), and an aggressive inflammatory environment (Thomas Hess, 2011). All of these are most likely to occur in long-established, chronic non-healing wounds. In other wounds such as acute surgical wounds or skin loss due to burns or injuries, there is a need to prevent fluid and electrolyte loss and bacterial colonisation of wounds. For burn wounds, the urgent need is to restore barrier function. Very superficial scalds can heal well and rapidly if managed conservatively. Deeper burns and full-thickness burns can be managed by the use of dermal substitutes such as Integra whose main function is to provide a well-vascularised dermal substrate to support skin grafts. This area has previously been reviewed in detail (MacNeil, 2007). Unfortunately, such sophisticated dressings are rarely available in some poorer countries. Hence in this study, we selected a dressing currently in use for both chronic non-healing wounds and burns wounds-specifically cotton fibre dressing and petrolatum wax coated cotton dressings, respectively.

A critical step in wound healing is the production of new blood vessels that deliver cells from the immune system to tackle infection and to breakdown damaged tissues. Blood vessels also provide metabolic support to the wound area via red blood cells that supply oxygen and growth factors to stimulate tissue regeneration. (Tonnesen, Feng, & Clark, 2000). Growth factors are released from the blood platelets, which aggregate in the wound area. This process is regulated via the production and release of a complex, highly-regulated series of growth factors that help to drive new blood vessel formation. Vascular endothelial growth factor (VEGF) is recognised as a critical growth factor. It is not only the most effective stimulator of angiogenesis but also helps to regulate many of the different aspects of angiogenesis at the molecular level (Ahluwalia & Tarnawski, 2012; Des Rieux et al., 2011; Roman et al., 2002; Witzenbichler et al., 1998). In addition to VEGF, there is platelet-derived growth factor (PDGF) and other pro-angiogenic growth factors which are generally produced on demand and bound to naturally occurring heparin from which they are cleaved as needed (Honnegowda et al., 2015). As expected, there have been extensive studies on the use of these well-established growth factors in wounds to accelerate healing (Nicosia, Nicosia, & Smith, 1994; Pierce, Tarpley, Yanagihara, Fox, & Thomason, 1992). Although their addition can accelerate wound healing, the results have proven modest rather than dramatic, and it is thought that this is due to the instability of these small peptides such that their biological actions do not last very long. The practical consequence of this is that growth factors need to be given every day or two days and indeed current research is now looking at gene therapy approaches to turn on the production of the growth factors when required (Öhnstedt et al., 2019).

Our group and several others have recently published that 2-deoxy sugars have unexpectedly been found to drive angiogenesis. This followed by the finding of a high level of 2 deoxy-D-Ribose (2dDR) in tumour angiogenesis (Nakajima, Madhyastha, & Maruyama, 2009). Accordingly, we defined the angiogenic concentrations of 2dDR *in vitro* (Dikici et al., 2020) and in *ex*-ovo CAM assay (Dikici, Mangır, Claeyssens, Yar, & MacNeil, 2019). Then, we further investigated whether this small stable sugar could be added to conventional wound dressings to drive angiogenesis. Our findings showed that it could be easily loaded to hydrogels made of chitosan (Yar et al., 2017) and alginate (Azam et al., 2019) and delivered from them to stimulate angiogenesis.

Although we have shown that 2dDR can be loaded into a variety of wound dressings, the challenge is to develop a dressing that is widely available, inexpensive, and can deliver 2dDR to stimulate angiogenesis and wound healing. Our study was based in Pakistan where, unfortunately, more sophisticated dressings are simply not available, and this is true for most of the less developed countries due to economic reasons. Surgeons trained in the West are frustrated that they cannot offer patients proactive dressings such as drug-releasing dressings or hydrogels or dermal substitutes such as Integra in these countries. Accordingly, in this study, we explored the potential of simple cotton fibre dressings in terms of delivering 2dDR. Throughout this study, we partnered with Pakistani surgeons and a Pakistani wound dressing company Cotton Craft. In addition to exploring the angiogenic potential of 2dDR loaded simple cotton dressings, cotton fibre dressings were also coated with medical-grade wax petrolatum to reduce the adherence of them to wounds (Dhivya, Padma, & Santhini, 2015; Olasupo Awe, 2019; Ramesh, Jayalakshmi, & Mohan, 2017). Cotton Craft has prior experience in combining established bioactives approved for clinical use with these petrolatum wax dressings (e.g. Parachlore Tulle Gras which delivers chlorhexidine acetate, a well-accepted antimicrobial disinfectant, and Poly Gras Dressing Surgical Tulle Dressing which delivers polymyxin B sulphate and Bacitracin Zinc, antibiotics to treat gram-negative and gram-positive infections, respectively).

Simple bandaging materials have good absorption capacities to absorb and trap exudates and act as a barrier to outside contaminants and microorganisms as infection prevention (Turner, 1979). Nonwoven fibrous wound dressings are considered an attractive option in the management of moderately and highly exudative wounds because of their absorption capacity, accessibility and low price (Dygai et al., 2011). On the other hand, clinicians usually recommend paraffin (wax)-coated dressings for partial-thickness burn wounds as they work well when used with a secondary dressing. These dressings act as a main wound contact layer, and the paraffin coating reduces the dressing adherence to the wound surface. Moreover, these dressings can effectively transfer the exudate from the wound surface through the perforations into the secondary dressing, and these perforations also allow one to inspect the wound through the dressing

(Hassanpour, Moosavizadeh, Yavari, Hallaj Mofrad, & Fadaei, 2013). Paraffin-coated gauze dressings are attractive candidates for the treatment of partial and full-thickness wounds burn wounds and are being used by plastic surgeons for the coverage of wounds as a standard skin graft donor site dressing (Olawoye, Ademola, Oluwatosin, Iyun, & Michael, 2017).

In this study, we used two types of commercially available cotton-based dressings: (i) cotton dressings covering a range of absorptive capacities and (ii) wax-coated cotton dressing. Several strategies were evaluated, and a straightforward methodology was selected for loading 2dDR into the wax-coated dressing that would be compatible with current manufacturing techniques. We assessed the uptake and release of 2dDR from these dressings over five days and were able to achieve approximately 90% release of sugar over five days from both types of dressing. We then investigated the pro-angiogenic activity of both the cotton fibre dressings and the cotton fibre wax dressing in the CAM assay and found that both were strongly pro-angiogenic. Quantification of angiogenesis showed that the increase in blood vessels formed over 7 days ranged from 50% when investigated macroscopically to 100% when investigated histologically, respectively. Our results demonstrated that loading 2dDR into commercially available cotton fibre dressings is a rapid, feasible, and effective way of driving angiogenesis in a well-accepted CAM bioassay.

2 | MATERIALS AND METHODS

2.1 | Materials

The cotton dressings (32 g/m^2, 40 g/m^2, 70 g/m^2, and 150 g/m^2), the cotton fibre (lino fabric) dressings, hard wax, beeswax, petroleum jelly, and liquid paraffin were provided by Cotton Craft Private Limited (Lahore, Pakistan). 2-Deoxy-D-ribose (2dDR) and Orcinol monohydrate ($CH_3C_6H_3$–1,3-$(OH)_2.H_2O$) were purchased from Sigma-Aldrich (China). Nutrient Agar broth was purchased from Microgen (India). Ethanol (99.8%) was purchased from Sigma-Aldrich (Germany). Ferric Chloride hexahydrate ($FeCl_3.6H_2O$) and hydrochloric acid (HCl) were purchased from AnalaR NORMAPUR (United Kingdom), and Merck (Germany) respectively. Glycerol was purchased from MERK Pakistan Limited. MTT Cell Proliferation Assay Kit was purchased from Bio Basic (USA).

2.2 | Methods

2.2.1 | Assessment of absorption capacities of different non-woven cotton dressings

The absorption capacities of different non-woven cotton dressings were determined by swelling them directly in PBS (phosphate buffer saline) solution (pH 7.4) at room temperature for 30 minutes. For this purpose, 20 × 20 mm^2 patches of different non-woven cotton dressings (32 g/m^2, 40 g/m^2, 70 g/m^2, 150 g/m^2) and cotton fibre dressings were used. The weight of the dressings was measured before immersion in PBS (dry weight) and after hydration in PBS (wet weight). Based on their swelling behaviour as well as the dressing's integrity 32 g/m^2, 40 g/m^2, 70 g/m^2 and 150 g/m^2 cotton dressings were selected for further studies.

2.2.2 | Assessment of loading of 2dDR into the cotton dressings

Pieces of selected non-woven cotton dressings of 20 × 20 mm^2 (32 g/m^2, 40 g/m^2, 70 g/m^2 and 150 g/m^2) were cut, autoclaved and placed at 37°C overnight to remove moisture. 1.85 mg of 2dDR (calculated as 5 wt% of dressing) prepared in sterilized PBS medium was dropped onto each sterilized dressing and allowed to air-dry in aseptic condition.

Loading of 2dDR and wax coating onto cotton dressings

The woven cotton dressings used in this study were lino fabric dressings in which cotton was processed into very thin fibres. The 20 × 20 mm^2 pieces of lino fabric dressings were cut, autoclaved and placed at 37°C overnight to remove moisture. Seven different strategies were carried out for introducing 2dDR into the wax coating to achieve a sugar release from wax-coated lino fabric dressing which will be called as cotton wax dressings throughout the manuscript. These strategies included the use of other carriers for sugar or the addition of an extra component in the wax mixture to decrease the sugar linkage with wax or by the surface coating of sugar onto the dressings. These strategies are explained below.

Strategy 1: 2dDR loading and wax coating using liquid paraffin as a sugar carrier. The aim of this protocol was that each 20 × 20 mm^2 piece of dressing should contain 1.85 mg of 2dDR (1.85 mg/20 × 20 mm^2 carrier dressing) as at this concentration, we have previously shown that 2dDR was effective in a diabetic rat model *(*Azam et al., *2019).* In the current strategy, two mixtures were prepared. **Mixture A** was prepared by heating the ingredients hard wax (1.15% w/w), beeswax (1.15% w/w) and petroleum jelly (97.7% w/w) at around 50°C temperature. This **mixture A** was used as a stock in other wax coating strategies given below. **Mixture B** was prepared by dissolving 18.5 mg of 2dDR into liquid paraffin (200 mg) by heating and vigorous stirring at around 65°C temperature. For the preparation of 10 pieces of 20 × 20 mm^2 dressings, **mixture B** (18.5 mg 2dDR in 200 mg liquid paraffin) was mixed with 500 mg of **mixture A** at around 50°C, and a homogenous final mixture was obtained. The 10 pieces of 20 × 20 mm^2 dressings were dipped into the final mixture and allowed to air-dry in aseptic conditions following the original industrial recipe of Cotton Craft PTV. LTD.

Strategy 2: Surface coating of the dressings with 2dDR using ethanol as a sugar carrier. In this strategy, the 10 sterile (autoclaved) 20 × 20 mm^2 pieces of cotton gauze dressings were taken and dipped into 500 mg

of **mixture A** (stock mixture prepared above in strategy 1) at 50°C and then allowed to air-dry in aseptic conditions to produce the wax-coated dressings. Then 2dDR (18.5 mg) was dissolved in ethanol (1,000 μl) (instead of liquid paraffin), and this was called as **mixture C**. The wax-coated 10 dressings were dipped into the **mixture C**, to achieve a surface coating of these dressings with 2dDR.

Strategy 3: Polyethylene glycol (PEG) addition using liquid paraffin as a sugar carrier. The methodology of Strategy 3 was similar to Strategy 1, but PEG was added to the sugar carrier solution (liquid paraffin). To achieve this PEG (100 mg) was dissolved in liquid paraffin (200 mg) by heating and continuous mixing, and then 2dDR (18.5 mg) was added to the mixture and dissolved via vigorous stirring at around 60°C temperature, this was called as the **mixture D**. The **mixture D** was then mixed with 500 mg of **mixture A** taken from the stock wax mixture as prepared above in Strategy 1. The rest of the procedure of dressing preparation and wax coating was performed following the same steps as Strategy 1.

Strategy 4: PEG addition and diluting the wax mixture to decrease 2dDR retention in the dressing. This strategy was similar to Strategy 3, except that the amount of liquid paraffin used was doubled (400 mg).

Strategy 5: Surface coating of sugar using liquid paraffin as a sugar carrier. In this strategy, 10 wax-coated dressings (20 × 20 mm^2) were prepared by dipping in 500 mg of **mixture A** (taken from the stock prepared in Strategy 1) at 50°C, and air-dried at room temperature. Then these dressings were dipped into **mixture B** as prepared in Strategy 1. Finally, they were allowed to air-dry in aseptic conditions.

Strategy 6: Addition of PEG and surface coating of sugar using liquid paraffin as a sugar carrier. This strategy was similar to Strategy 5. The only difference was that the prepared wax-coated dressings were dipped into **mixture D** as prepared in Strategy 3 instead of **mixture B**.

Strategy 7: Diluting the wax mixture to decrease 2dDR entrapment in the dressing. In this strategy, the overall method was the same as for Strategy 1 (original industrial recipe), but the amount of liquid paraffin was doubled to 400 mg. In this way, overall, a 20 × 20 mm^2 wax-coated dressing took up an average of 1.85 mg 2dDR. All further steps of the wax coating were performed as in Strategy 1.

2.2.3 | Assessment of release of 2dDR from dressings

2dDR release from non-woven cotton dressings

The accumulative release of 2dDR from 32 g/m^2, 40 g/m^2, 70 g/m^2 and 150 g/m^2 cotton dressings was determined using Bial's orcinol assay. The sugar-loaded 20 × 20 mm^2 pieces of the dressings were immersed in 4 ml of PBS solution and incubated at 37°C. The medium was collected and replaced by fresh PBS after 4 hours and afterwards daily until the medium showed no significant drug release. Bial's assay was performed to confirm 2dDR presence in the collected medium as described previously (Patterson & Mura, 2013). Briefly, 4 ml volume of the collected sample solution and the same amount of Bial's reagent (a solution of orcinol, HCl and ferric chloride) was placed in falcon tubes. The solution was incubated in a boiling water bath for 20 minutes. The heated sample was then removed from the water bath and allowed to cool at room temperature. The sample tubes were centrifuged to sediment any particulate material and absorbance was measured at 630 nm using a UV–VIS spectrophotometer. Absorbance values were converted into concentrations using a standard curve of known concentrations of 2dDR.

Following opinions received from the plastic surgeons with whom we are collaborating, the 150 g/m2 cotton dressing from among the non-woven dressings was selected for further studies. The 150 g/m^2 cotton dressing was chosen for its use in chronic and exuding wounds because of its fabric integrity, smoothness, lightweight, high strength and best absorption capacity.

2dDR release from cotton wax dressings

The accumulative release of 2dDR over 5 days from wax-coated dressings (gamma sterilized at 25 kG) of all sugar loading strategy groups was determined using Bial's orcinol assay as given above.

2.2.4 | The effect of glycerol treatment on the release profile of 2dDR

After the absorption of wound exudate, the dressing may become dry, which would disturb the peri-wound area and cause pain to the patient. We aimed to minimize the dressing roughness after absorption of wound exudate through the addition of glycerol onto wax-coated dressings. The effect of glycerol coating on the rate of 2dDR release from dressings was also determined. Briefly, the effect of glycerol on moisture retention and sugar release from wax-coated dressings were studied by treating the dressings with 4% glycerol solution (prepared in distilled water). For this purpose, 20 × 20 mm^2 patches of wax-coated dressings (n = 6) were prepared according to strategy 7. Half of the dressings were treated with glycerol (glycerol treated group) while the other half were taken as the control group. For glycerol treatment, 5% 2dDR loaded cotton wax dressings were dipped in 4% glycerol solution and allowed to dry in a sterile environment. Sugar release studies from both the groups (control and glycerol treated) were performed using Bial's orcinol assay as previously described, and the difference in sugar release behaviour from both the groups was calculated.

2.2.5 | Sterilization Test

The sterility of the 5% 2dDR loaded 150 g/m^2 cotton dressings and 5% 2dDR loaded cotton wax dressings was ensured by performing diffusion disk tests before and after gamma sterilization at 25 kG. For this purpose, 5% 2dDR loaded patches with and without gamma

sterilization from both dressing groups were prepared. The study was divided into two sections (5% 2dDR loaded 150 g/m^2 cotton dressing and 5% 2dDR loaded cotton wax dressing) each having three groups (control, dressings without gamma sterilization and dressings after gamma sterilization). Briefly, nutrient broth medium and nutrient agar medium were prepared and autoclaved. 10 ml of nutrient broth medium was taken in test tubes, dressings from both sections were immersed in it and placed at 37°C for 24 hours. The experiment was performed in triplicate, and a petri dish with broth medium without any dressing was used as a control. After 24 hours incubation at 37°C, 250 μl of broth medium was taken from all groups and spread on agar plates and left overnight at 37°C. The agar plates were carefully examined for microbial growth after 24 hours and were compared for the sterilities of the dressings.

2.2.6 | Cytotoxicity testing of 2dDR loaded 150 g/m^2 dressings using MTT assay

To evaluate the cytotoxicity of 150 g/m^2 cotton dressings, NIH/3 T3 mouse fibroblast cell lines were used. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed in which dressings were placed in indirect contact with fibroblasts using transwell plates. Briefly, fibroblasts were seeded into the base of 24 well transwell plates at a density of 2×10^4 cells/well in 15% FBS supplemented high glucose Dulbecco's Modified Eagle Medium (DMEM-HG), and the plates were incubated in a 5% CO2 humidified incubator at 37°C. *gamma* sterilized dressings were placed above the cells in the transwell plates so that they were submerged in the same media as the cells but not in direct contact. The culture media was regularly changed every 2 days throughout the experiment duration. The metabolic activities of fibroblasts in indirect contact with all 3 groups (tissue culture plastic (TCP), 150 g/m^2 control dressing, 5% 2dDR containing 150 g/m^2 cotton dressing) at day 3 and day 7 were examined by MTT assay (MTT cell proliferation assay kit, Bio Base, USA) according to the manufacturer instructions (Wiegand, Abel, Hipler, & Elsner, 2019). The optical density of each group was recorded at a wavelength of 630 nm using an ELISA (Biorad) plate reader. DMSO was taken as standard. The results for each group were compared by statistical analysis.

2.2.7 | Assessment of the angiogenic potential of dressings using the chick chorioallantoic membrane (CAM) assay

Angiogenic activity of 5% 2dDR loaded 150 g/m^2 cotton dressing

The angiogenic activity of 5% 2dDR loaded 150 g/m^2 cotton dressing was evaluated using the CAM assay over 7 days (Mangir, Dikici, Claeyssens, & MacNeil, 2019), and non-loaded 150 g/m^2 cotton dressing was used as a control throughout the experiments. Ten embryos were used for each group, and the experiment was carried out under sterile conditions. Briefly, fertilized chicken eggs were purchased from Big Bird Industry (Lahore, Pakistan) and incubated at 37°C in a humidified egg incubator. At day 7, an area of about 1 cm^2 in the form of a square window was cut and removed from the shell. Less than 1 cm^2 pieces of dressings from both groups were cut and implanted onto the CAM by inserting these through the square window. The shell window was covered with parafilm (disinfected by immersing into 70% ethanol prior to use) and sealed with adhesive tape. After implantation, eggs were returned to a humidified egg incubator and incubated at 37°C until day 14. At day 14, the shell windows were opened carefully, images were captured using camera and dressings were retrieved for further evaluation.

Angiogenic activity of 5% 2dDR loaded cotton wax dressing

B2 twb=.4w?>The angiogenic potential of 5% 2dDR loaded cotton wax dressings was assessed using the same protocol as described above.

2.2.8 | Histological evaluation of the cotton dressings

The dressings were histologically evaluated using hematoxylin–eosin staining (H&E) of the sections retrieved from either cotton dressings (non-loaded or loaded with 5% of 2dDR) or cotton wax dressings (non-loaded or loaded with 5% of 2dDR). In brief, the retrieved dressings from the CAM containing newly formed vascular networks were fixed in 10% paraformaldehyde solution and embedded in paraffin for block preparation. The paraffin blocks were sectioned using a microtome at a thickness of 5 μm, and the sections were stained with H&E, as described previously (Aldemir Dikici, Dikici, Reilly, MacNeil, & Claeyssens, 2019).

2.2.9 | Quantification of angiogenesis

For quantification of the angiogenesis from macroscopic images of the blood vessels, a semi-quantitative assessment method was performed by observers who were blind to the samples presented to them using a qualitative grading scale: 0 = not angiogenic, 1 = weakly angiogenic, 2 = mildly angiogenic, 3 = moderately angiogenic, 4 = highly angiogenic, 5 = very highly angiogenic. At least seven embryos were used for each group studied. Representative images of the dressings were obtained from all seven embryos and were assessed and scored by five researchers who were not informed of the conditions used in each embryo.

For quantification of the angiogenesis from histological staining of the sections, images were taken with a digital camera coupled to the microscope and were analysed by rating the extent of blood vessels formed in each group. The total number of blood vessels adjacent to the dressings were quantified by counting blood vessels in H&E sections, as described previously (Dikici, Mangır,

et al., 2019; Mangir et al., 2019). Briefly, all discernible blood vessels adjacent to the dressings were counted using a light microscope at 10 × magnification.

2.2.10 | Statistical analysis

Statistical analyses were performed by one-way analysis of variance (ANOVA) test followed by unpaired Bonferroni test. For the analysis of the CAM assay results, unpaired t-test with Welch's correction was used. $P < 0.05$ was considered to be statistically significant, and the data were expressed as means ± SD.

3 | RESULTS

3.1 | Swelling properties of different non-woven dressings

Swelling studies of non-woven cotton dressings (32 g/m^2, 40 g/m^2, 70 g/m^2, and 150 g/m^2) and cotton fibre dressings were carried out by immersing them directly in PBS for 30 minutes, and by measuring their wet weight. The appearance of the dressing's pre and post liquid absorption is shown in Figure 1a. All the dressings showed good absorption capacity, whereas cotton fibres and 150 g/m^2 took up considerably more liquid than the others, as shown in Figure 1b. Based on their integrity, smoothness and their state when wet, 32 g/m^2, 40 g/m^2, 70 g/m^2 and 150 g/m^2 cotton dressings were selected for drug release studies. The simple cotton fibre dressing was excluded because of its loose fibrous nature which may cause undesired attachment to the wound.

Figure 2 shows the pictures of 150 g/m2 cotton dressing and cotton wax dressings loaded with 5% 2dDR showing their flexibilities and abilities to be folded.

3.2 | Release of 2dDR from non-woven cotton dressings

A representative image that shows the qualitative evaluation of 2dDR release over 5 days from 150 g/m^2 cotton dressing, which was selected for further studies is given in Fig. 3 Aa. The blue colour indicates the presence of 2dDR in the solution. The change of colour from blue to yellow over 5 days demonstrated that there was less release of sugar after this point (Fig. 3 Aa). The graph in Fig. 3 Ab shows the cumulative release of 5% 2dDR (1.85 mg/20 × 20 mm^2 patches) loaded into 32 g/m^2, 40 g/m^2, 70 g/m^2, and 150 g/m^2 non-woven cotton dressings. The *in vitro* sugar release profile showed that 32 g/m^2 cotton dressing gave an average sugar release of 255 μg/day whereas the release of 2dDR was 616 μg/day and 566 μg/day from 40 g/m^2 and 70 g/m^2 cotton dressings, respectively. For all of the dressings given above (32 g/m^2, 40 g/m^2, and 70 g/m^2), almost all (>90%) of the loaded sugar was released within 3 days. On the other hand, 150 g/m^2 cotton dressing showed a more sustained release profile of 2dDR over 5 days with an average release of 370 μg/day.

3.3 | Release of 2dDR from cotton wax dressings

Fig. 3 Ba shows the cumulative release of 2dDR (1.85 mg/20 × 20 mm^2 patches) loaded into wax-coated cotton dressings using different strategies. The *in vitro* drug release profile showed that in strategy 1 (the

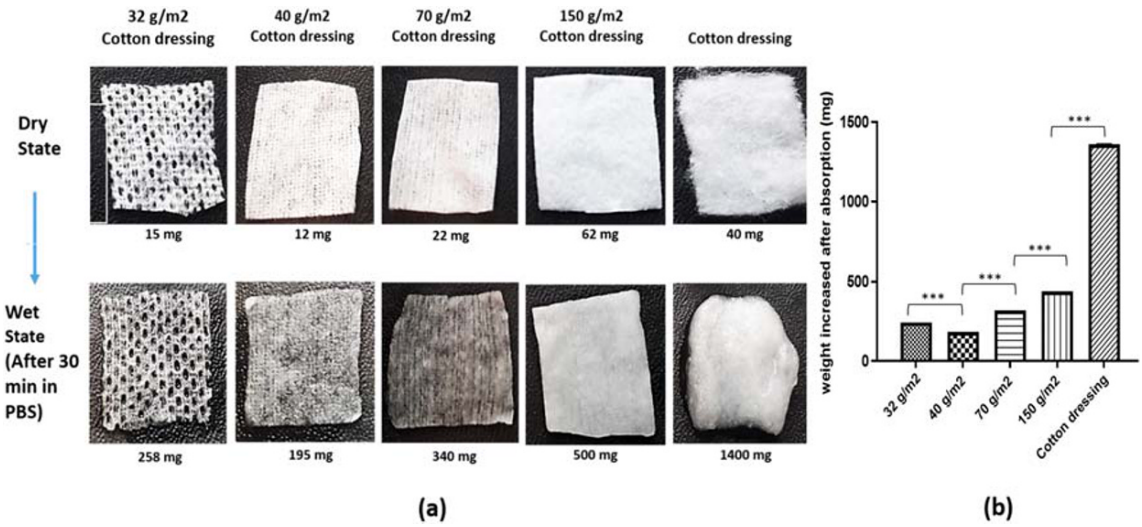

FIGURE 1 Figure (a) shows swelling studies of different non-woven cotton dressings (32 g/m^2, 40 g/m^2, 70 g/m^2, and 150 g/m^2) and cotton fibre dressings. The figure shows their appearance before and after immersion in PBS for 30 minutes. Based on integrity, smoothness and their wet state 32 g/m^2, 40 g/m^2, 70 g/m^2, and 150 g/m^2 cotton dressings were selected for further studies. Figure (b) shows a graphical representation of the extent of weight gained by these dressings after PBS absorption. *** $P \leq 0.001$, $n = 3 \pm$ SD [Colour figure can be viewed at wileyonlinelibrary.com]

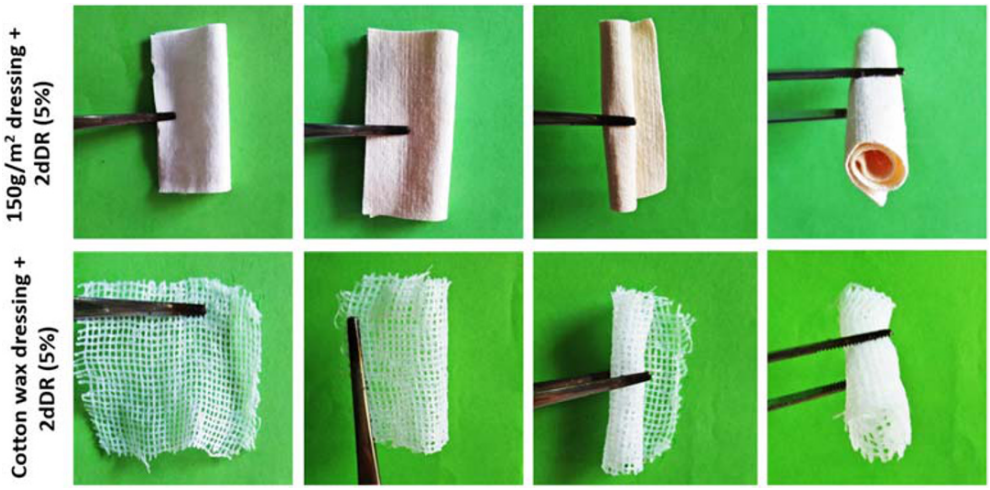

FIGURE 2 Pictures of 150 g/m^2 cotton dressing and cotton wax dressings loaded with 5% 2dDR showing their flexibility and ability to be folded [Colour figure can be viewed at wileyonlinelibrary.com]

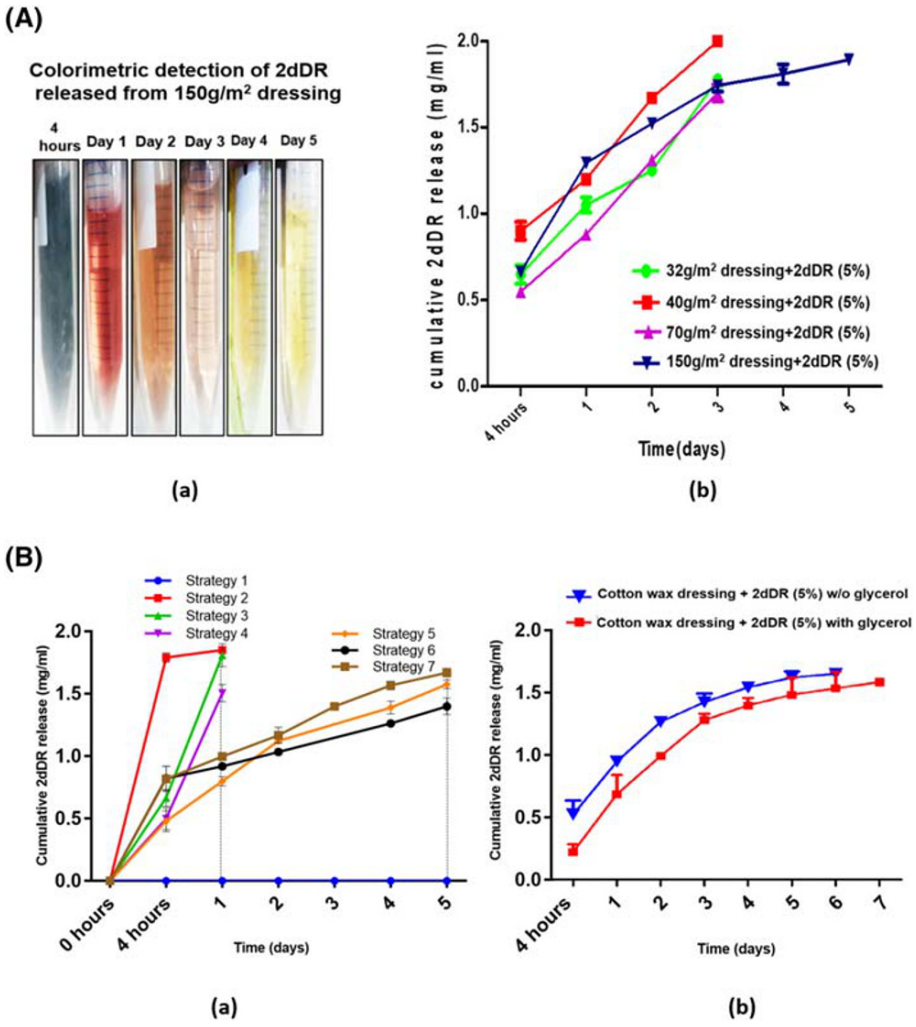

FIGURE 3 The release of 2dDR was assessed using Bial's orcinol assay is shown in Figure (A). Figure (Aa) shows colorimetric detection of 2dDR released from 150 g/m^2 + 2dDR (5%) dressing to the medium using Bial's orcinol assay and (Ab) shows the cumulative release of 2dDR (1.85 mg/20 × 20 mm^2 patches) from different non-woven cotton dressings (32 g/m^2 dressing + 2dDR (5%), 40 g/m^2 dressing + 2dDR (5%), 70 g/m^2 dressing + 2dDR (5%), and 150 g/m^2 dressing + 2dDR (5%)). The release profiles from wax-coated cotton fibre dressings are given in Figure (B). Figure (Ba) shows the cumulative release of 2dDR from cotton fibre dressings prepared using different strategies. Strategy 2,3, and 4 showed a similar release behaviour which was very quick in 1 day while strategy 5,6, and 7 gave a more sustained release pattern in 5 days. No release was observed from dressings wax-coated using strategy 1. Figure (Bb) shows the comparison of 2dDR release from 5% 2dDR loaded cotton wax dressings with and without glycerol treatment [Colour figure can be viewed at wileyonlinelibrary.com]

original industrial recipe), no sugar release was observed from the dressings until at least 7 days. When strategy 2 (surface coating of sugar using ethanol as a drug carrier) and strategy 3 (PEG addition in the original recipe for wax coating) were used for loading 2dDR, an average sugar release of 1850 μg/day and 1800 μg/day was observed, respectively. In strategy 4 (PEG addition in the diluted wax mixture), the average drug release was slightly less, and it was measured as 1,500 μg/day. The use of strategy 5 (surface coating of sugar using liquid paraffin as a drug carrier) and strategy 6 (PEG addition and surface coating of sugar using liquid paraffin as a drug carrier) resulted in a comparably lower daily 2dDR release profile with an average of 314 μg/day and 280 μg/day, respectively. Similar to strategy 5 and 6, in strategy 7 (diluting the wax mixture to decrease 2dDR entrapment), an average drug release of 334 μg/day was measured. The 2dDR release

TABLE 1 Strategies for loading 2dDR into cotton wax dressings. The number of "+" indicates the effectiveness of the respective strategy when the ease of manufacturing and the release behaviour were considered. "+++" = very effective, "++" = effective, "+" = less effective, and "-" = not effective

Strategy	Ingredients	Loading Method	Release Duration	Release (μg/day)	Reasons for rejection/selection	Effectiveness
1	Wax mixture Liquid paraffin 2dDR	2dDR was dissolved in liquid paraffin and mixed into melted wax mixture	No release over 5 days	0	No 2dDR release was observed over 5 days	–
2	Wax mixture Ethanol 2dDR	Surface coating of 2dDR using ethanol as a sugar carrier	1 day	1850	The release of all the loaded 2dDR was too quick	+
3	Wax mixture Liquid paraffin PEG 2dDR	PEG was added to liquid paraffin as a sugar carrier	1 day	1800	The release of all the loaded 2dDR was too quick	+
4	Wax mixture Liquid paraffin PEG 2dDR	PEG was added to increased quantity of liquid paraffin as a sugar carrier	1 day	1,500	The release of all the loaded 2dDR was too quick	+
5	Wax mixture Liquid paraffin 2dDR	Surface coating of 2dDR using liquid paraffin as a sugar carrier	5 days	314	Good sustained release profile was obtained, but it required two coating steps	++
6	Wax mixture Liquid paraffin PEG 2dDR	Surface coating of 2dDR using PEG added liquid paraffin as a sugar carrier	5 days	280	Good sustained release profile was obtained, but it required two coating steps	++
7	Wax mixture Liquid paraffin 2dDR	Diluted wax mixture was used by increasing the amount of liquid paraffin (sugar carrier)	5 days	334	Good sustained release profile was obtained using a coating protocol corresponding to the original recipe used in industry	+++

behaviours of the wax-coated cotton dressings using different strategies are summarised in Table 1.

In strategies 2, 3 and 4, almost all of the loaded 2dDR (>90%) was released within 1 day (in 24 hours). As a sustained release is required from these dressings to stimulate wounds, these strategies were excluded from future experiments. With the use of strategy 5, a better release profile was obtained, and the loaded sugar was released throughout 5 days. However, this strategy was also excluded because it is more complicated for industry to manage one setup for wax coating and a separate setup for the sugar coating of dressings. Similarly, strategy 6 gave a good release behaviour over 5 days, but this strategy was also excluded due to the complexity of introducing it to industry and secondly, it requires the addition of an extra component of PEG to stimulate sugar release.

Finally, strategy 7 was found to be the best strategy of these protocols because of its sustained sugar release over 5 days, and it follows the same production procedure as the original industrial recipe without the addition of any extra components such as PEG or ethanol to achieve a better sugar release profile from the dressing.

3.4 | The efficiency of glycerol treatment on moisture retention and sugar release profile in 2dDR loaded cotton wax dressing

The effect of glycerol treatment on the release behaviour of loaded sugar from the cotton wax dressings was evaluated by treatment with 4% glycerol solution prepared in distilled water. The evaluation of the release profile of 2dDR from cotton wax dressings either non-treated or treated with glycerol demonstrated that the glycerol treatment of the 5% 2dDR loaded cotton wax dressings resulted in slightly slower release of sugar (non-significant) over 6 days compared to 5% 2dDR loaded cotton wax dressings which were not treated with glycerol as shown in Fig. 3Bb.

3.5 | Confirmation of the sterility of 150 g/m^2 cotton dressing and cotton wax dressing

To confirm the sterility of the gamma sterilised 5% 2dDR loaded 150 g/m^2 cotton dressing and 5% 2dDR loaded cotton wax dressing, these were incubated in Nutrient broth for 24 hours. In the case of the sterility test performed on dressings without gamma sterilization, microbial growth was observed on the agar plates after 24 hours from both dressing groups when compared with the control, which indicated that the dressings were not sterile. The sterilisation of the dressings after gamma treatment was verified as no bacterial growth was observed on the agar plates after 24 hours incubation at 37°C (Figure 4a).

3.6 | Cytotoxicity testing of 150 g/m^2 dressing using MTT assay

The MTT assay results performed using 150 g/m^2 cotton dressing are shown in Figure 4b. The dressings did not show any evidence of

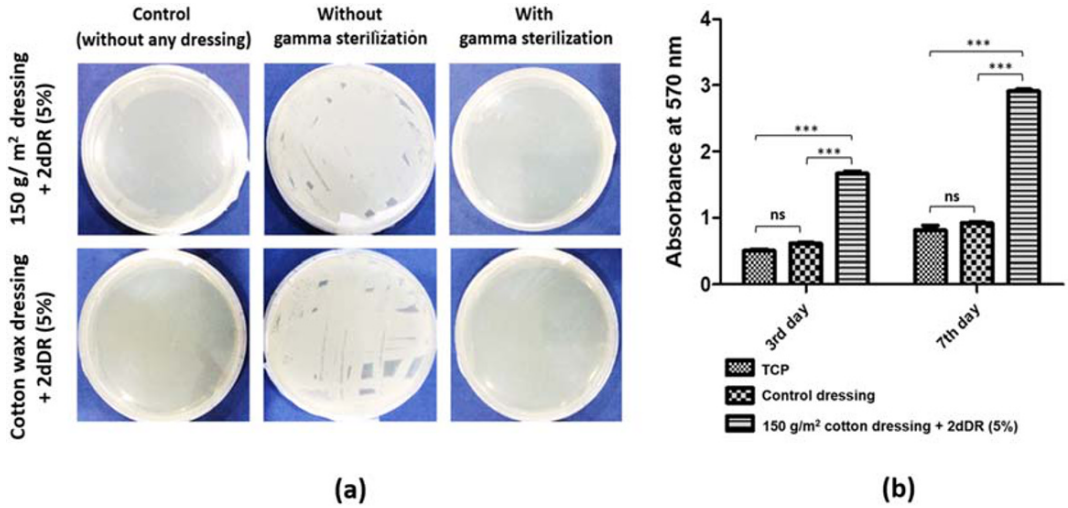

FIGURE 4 Figure (a) shows the sterility testing of 5% 2dDR loaded 150 g/m^2 cotton dressing and cotton wax dressing using Nutrient Agar plates. The figure shows the sterility test of control (simple broth used without any dressing), sterility test of dressings without gamma sterilization and after irradiation with gamma rays. In case of dressings without gamma sterilization, the agar plates showed contamination due to microbial growth, whereas no microbial growth was found on the agar plates when gamma sterilized dressings were used. Figure (b) shows the assessment of cytotoxicity of dressings using MTT assay performed on 150 g/m^2 cotton dressing. The graph shows the extent of cell viability in TCP (simple cells plated on tissue culture plate without any dressing), control dressing (150 g/m^2 cotton dressing without 2dDR) and 5% 2dDR loaded 150 g/m^2 cotton dressing groups at 3rd day and 7th day. "ns" represent non-significance in cotton dressing group vs TCP after both 3rd day and 7th day. ***P ≤ 0.001 in 5% 2dDR loaded 150 g/m^2 cotton dressing group vs TCP and control dressing after both 3rd day and 7th day. Results presented as mean ± SD, n = 3 [Colour figure can be viewed at wileyonlinelibrary.com]

cytotoxicity. Indeed, cells showed higher metabolic activity when cultured in indirect contact with the 5% 2dDR loaded 150 g/m^2 cotton dressings group compared to other groups. MTT assay results at both days 3 and 7 demonstrated higher cell viability with sugar-containing dressing compared to control. The results suggested that the released 2dDR over several days gradually increased cell proliferation.

3.7 | Assessment of the angiogenic potential of dressings using the chick chorioallantoic membrane (CAM) assay

Figure 5A shows that 5% 2dDR loaded 150 g/m^2 cotton dressing stimulated a much greater degree of angiogenesis compared to the dressing without 2dDR, with a rich vascular network surrounding the dressing placed on the CAM.

Similarly, the 5% 2dDR loaded cotton wax dressing induced significantly more blood vessels as compared to the simple gauze dressing without wax and 2dDR. Macro images and graphical representations of the extent of angiogenesis for both groups are shown in Figure 5B.

The results of the H&E staining of CAMs treated with the 150 g/m^2 cotton dressing, and cotton wax dressing are given in Figure 6. The control dressing without 2dDR showed only a few blood vessels (Figure 6A) close to the area of implantation whereas the 5% 2dDR loaded 150 g/m^2 cotton dressing induced a large number of blood vessels with blood cells and inflammatory cells within them as shown in Fig. 6Ab and 6Ac. Moreover, a dense connective tissue network was seen in the 2dDR containing group as compared to the control group. Graphical representation of the number of vessels detected in H&E stained images from both groups is shown in Fig. 6A. Blood vessels are indicated with red-headed arrows in the stained images.

The addition of 5% 2dDR to cotton wax dressings induced the formation of more blood vessels, as shown in Figure 6b compared to its respective control dressing (Fig. 6 Ba). Fig. 6 BC shows the graphical representation of the number of vessels present in H&E stained images from both groups.

5% 2dDR loaded 150 g/m^2 cotton dressing resulted in increased angiogenesis on the CAM compared to the 5% 2dDR loaded cotton wax dressing this was most evident when the extent of new blood vessels was determined through histological evaluation of the cotton dressings retrieved from the CAM as shown in Figure 6.

4 | DISCUSSION

Wounds are complex in nature, and no single universal dressing is available, which can treat all types of wounds effectively (Rajendran, 2010). The selection of the most appropriate wound dressing for a particular wound not only depends on its type but also on its stage, and more importantly, it requires clinical assessment and specialist recommendation and knowledge (Uzun, 2018). Many medical dressings are expected to possess hydrophilic properties with enhanced ability to take up wound fluid (Lionelli & Lawrence, 2003). The dressings with good sorption capacity are

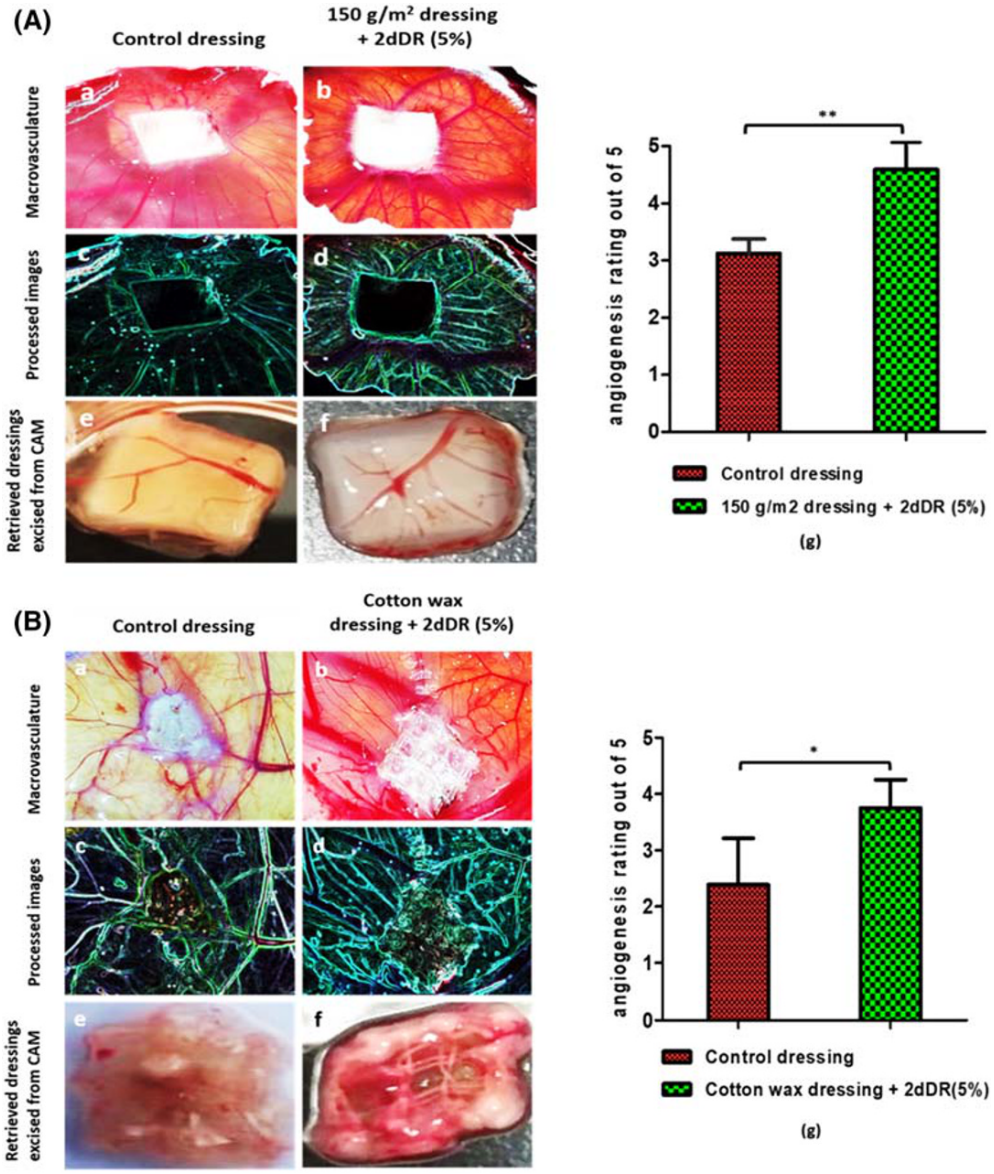

FIGURE 5 Assessment of the angiogenic potential of 5% 2dDR loaded 150 g/m^2 cotton dressing and cotton wax dressing using the CAM assay: The dressings were placed into the fertilized eggs at day 7 of fertilization. The figure (A) shows the pictorial illustration of control dressing (150 g/m^2 cotton dressing without sugar) and 5% 2dDR loaded 150 g/m^2 cotton dressing on the embryo at day 14 of fertilization. The picture (Aa, Ab) shows the original CAM macro-vasculature, (Ac, Ad) shows their respective processed images using image J. and (Ae, Af) shows the respective explanted dressings. The figure (Ag) shows the graphical representation of the rate of angiogenesis for both control dressing and 5% 2dDR loaded 150 g/m^2 cotton dressing using T-test analysis. Similarly, the figure (B) shows the pictorial illustration of cotton wax dressing (control dressing) and 5% 2dDR loaded cotton wax dressing on the embryo at day 14 of fertilization. The picture (Ba, Bb) shows the original CAM macro-vasculature, (Bc, Bd) shows their respective processed images using image J. and (Be, Bf) shows the respective explanted dressings. The figure (Bg) shows the graphical representation of the rate of angiogenesis out of 5 for both control dressing and 5% 2dDR loaded cotton wax dressing using T-test analysis. A rich vascular network was observed in the groups treated with 5% 2dDR loaded 150 g/m^2 cotton dressing and cotton wax dressing as compared to their respective control dressings. However, 2dDR loaded 150 g/m^2 cotton dressing have shown significantly more angiogenesis as compared to 2dDR loaded cotton wax dressing. Results presented as mean ± SD, n = 8 [Colour figure can be viewed at wileyonlinelibrary.com]

considered more suitable for chronic wounds, including diabetic wounds and burn ulcers, where exudate production is a major issue (Cullen, Smith, Mcculloch, Silcock, & Morrison, 2002). In the current study, all the non-woven cotton wound dressings (32 g/m^2, 40 g/m^2, 70 g/m^2, and 150 g/m^2) showed good absorption capacity as depicted by their swelling behaviour in PBS in 30 minutes. In addition, 150 g/m^2 cotton dressing, not only demonstrated good absorption capacity, but also had a smooth surface, and the dressing was more resilient to handling compared to other cotton dressings. Because of its ability to take up liquid efficiently, this dressing could have the potential to be used for chronic exudative wounds.

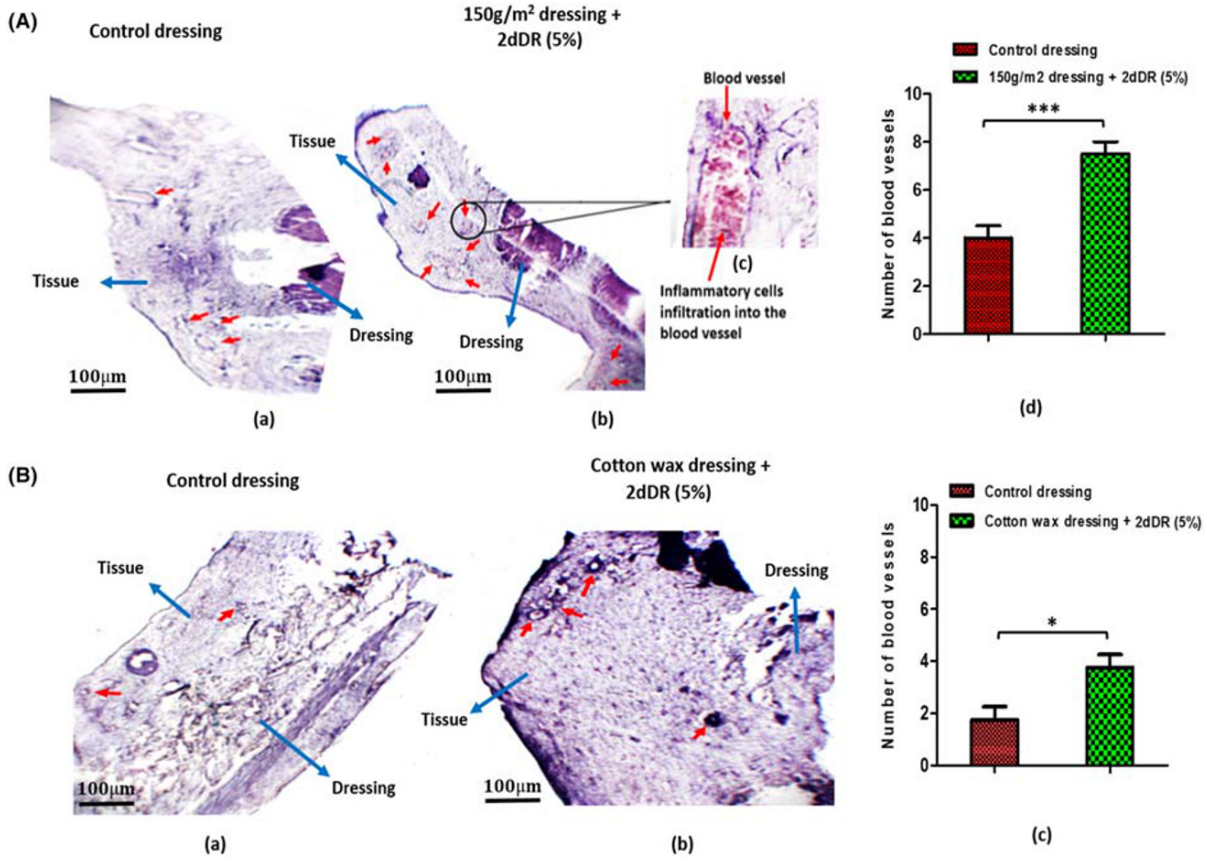

FIGURE 6 Histological analysis of CAM assay retrieved dressings: The figure (Aa) shows hematoxylin–eosin staining of control dressing and (Ab) shows staining of 5% 2dDR loaded 150 g/m^2 cotton dressing, (c) shows cross-section of image depicting the blood vessel containing red blood cells along with infiltrated inflammatory cells (infiltration of inflammatory cells occur from connective tissue bed into the blood vessels) while (Ad) shows graphical representation of comparison of number of blood vessels present in the H&E stained images from both 150 g/m^2 cotton dressing and 5% 2dDR loaded 150 g/m^2 cotton dressing using T-test analysis. Similarly, the figure (Ba) shows hematoxylin–eosin staining of control dressing, (Bb) shows staining of 5% 2dDR loaded cotton wax dressing and (Bc) shows the graphical representation of the comparison of number of blood vessels present in the H&E stained images from both control dressing and 5% 2dDR loaded cotton wax dressing using T-test analysis. A considerable increase in blood vessel formation (shown by redheaded arrows) was observed in the groups treated with 5% 2dDR loaded 150 g/m^2 cotton dressing and cotton wax dressing as compared to their respective control dressings. However, 2dDR loaded 150 g/m^2 cotton dressing have resulted in more blood vessels as compared to 2dDR loaded cotton wax dressing. Results presented as mean ± SD, $n = 6$ [Colour figure can be viewed at wileyonlinelibrary.com]

Burn wounds are challenging to manage because they can be highly exudative and often have a large surface area. Burn patients also experience a lot of pain if dressings adhere to the wound bed, and this may cause secondary trauma when the dressings are removed (Gosselin & Kuppers, 2008; Kim et al., 2014). Wax-coated dressings have been widely used as burn wound dressing materials due to their smooth texture which reduces adhesion to the wound bed (Thomas, 1994). Various conventional gauze dressings such as Jelonet (Smith & Nephew Healthcare Limited, Watford, UK), Adaptic (Johnson & Johnson Medical, New Jersey, United States), and Mepitel (Mölnlycke Health Care, Gothenburg, Sweden) with perforated or open mesh structure have long been used for the treatment of burn wounds (Hassanpour et al., 2013). A clinical study reported the efficacy of paraffin gauze dressings in comparison with dry mesh gauze dressings and reported that the former resulted in more rapid and complete re-epithelialization (Martini, Reali, Borgognoni, Brandani, & Andriessen, 1999). Moreover, patients with paraffin gauze dressings reported less pain during dressing removal than when treated with dry mesh gauze dressings (Weber et al., 1995).

Accordingly, in this study, we compared seven wax coating strategies for introducing 2dDR into the wax-coated cotton dressings for wounds that require a treatment where less-adherent wound dressings are used. Our findings showed that strategy 7, a strategy very similar to the original commercial wax coating methodology (protected as proprietary property of Cotton Craft), was found to be the most effective method when the ease of manufacturing and the release behaviour were considered.

Angiogenesis is an essential stage of the normal wound healing process, but chronic wounds have impaired angiogenesis because of inadequate blood supply hence less local growth factors which are crucial to driving angiogenesis. This results in delayed wound healing (Honnegowda et al., 2015). Promoting neovascularisation in the

wound area can be induced by the local delivery of growth factors, chemical mediators and endothelial cell chemoattractants (Tonnesen et al., 2000). Amongst these approaches, the use of pro-angiogenic agents is widely-studied, and many researchers have tried to manipulate these pro-angiogenic components to improve healing in experimental wound models (Hoeben et al., 2004).

VEGF is recognised as a critical factor in promoting angiogenesis (Hoeben et al., 2004). ECs are sensitive to VEGF signalling, which mediates proliferation, migration and survival of them (Olsson, Dimberg, Kreuger, & Claesson-Welsh, 2006; Wang et al., 2008). However, despite the effectiveness of VEGF in inducing angiogenesis, the use of exogenous VEGF in an uncontrolled manner has previously been reported to result in the promotion of leaky (Yancopoulos et al., 2000), permeable (Cao et al., 2004) and haemorrhagic (Cheng, Nagane, Huang, & Cavenee, 1997) blood vessels. Thus, the use of alternative pro-angiogenic agents to ensure neovascularisation in wound healing is essential.

2dDR is one of the degradation products of thymidine to thymine, which is produced *in vivo* by the catalytic action of thymidine phosphorylase (TP), and it has previously been reported as an angiogenesis-inducing factor *in vitro* and *in vivo* (Sengupta, Sellers, Matheson, & Fan, 2003) and an endothelial-cell chemoattractant (Brown & Bicknell, 1998). As a local delivery approach, the pro-angiogenic potential of 2dDR has recently been reported by our group *in vitro* (Dikici, Aldemir Dikici, et al., 2020), in an *ex ovo* CAM bioassay (Dikici, Mangır, et al., 2019), and in the acceleration of wound healing in both normal wounds (Yar et al., 2017) and diabetic wounds in rats (Azam et al., 2019). Although the mechanism of action of 2dDR is still unclear, the current literature focuses on two potential pathways: (i) the intracellular production of 2dDR by degradation of thymidine to thymine induces oxidative stress and stimulates the secretion of angiogenic factors such as VEGF and interleukin-8 to promote angiogenesis (Brown, Jones, Fujiyama, Harris, & Bicknell, 2000; Nakajima et al., 2009; Sengupta et al., 2003) and (ii) 2dDR activates NADPH oxidase 2 which triggers the activation of nuclear factor-kappa B to upregulate the VEGF receptor 2 expression (Vara et al., 2018). Previous studies of our group demonstrated that 2dDR is an effective and inexpensive alternative to VEGF for promoting angiogenesis. Our prior works exploring 2dDR for promoting angiogenesis and wound healing are summarised in Table 2.

In the present work, 2dDR is added to commercially available cotton-based dressings. The 2dDR release studies from non-woven cotton dressings showed that the sugar release profile from 32 g/m^2, 40 g/m^2, and 70 g/m^2 cotton dressings was rapid with almost all the loaded sugar (>90%) released from the dressings within 3 days. This is an expected outcome since the dressings with lower weights per square meter have a relatively higher surface area where 2dDR is released from, as can be seen in Figure 1. An average release of 592 μg/day, 660 μg/day, and 566 μg/day were observed over 3 days from 32 g/m^2, 40 g/m^2, and 70 g/m^2 cotton dressing, respectively. The 2dDR release behaviour from 150 g/m^2 cotton dressing was more sustained up to 5 days. This is more likely due to having a lower liquid contact area as the weight of this dressing per square meter area is higher than the others. However, the burst release of 2dDR was observed in 2–3 days (the average release of 2dDR was 370 μg/day). We have previously demonstrated that a similar release profile of 2dDR from commercially available alginate dressing not only stimulated angiogenesis but also improved wound healing in diabetic rats (Azam et al., 2019). In addition, a very similar release behaviour has previously been observed when 2dDR was released from electrospun polymer nanofibres to stimulate angiogenesis in *ex-ovo* CAM assay (Dikici, Mangır, et al., 2019). This release pattern is appropriate for wounds where the dressing is changed once or twice a week (Saghazadeh et al., 2018).

The release profiles from cotton wax dressings prepared using different sugar loading strategies are summarised in Table 1. The different strategies to load 2dDR into wax-coated cotton gauze dressings has resulted in different release behaviour of 2dDR because of different components used in the procedures. In strategy 1 (original industrial recipe), no drug release was observed because 2dDR was tightly entrapped in highly viscous wax coating on the dressings. In strategy 2, when the wax-coated cotton dressing was dipped into

TABLE 2 A summary of our prior works exploring the use of 2dDR for promoting angiogenesis and wound healing. "n/a" corresponds to not applicable

Assay	The type of carrier explored	Effective doses of 2dDR	Result	References
In vitro	n/a	100 μM to 1 mM	Promotes endothelial proliferation, migration and tube formation	(Dikici, Aldemir Dikici, et al., 2020)
In-ovo CAM	Chitosan/collagen hydrogel	1 mg/ml	Promotes angiogenesis	(Yar et al., *2017*)
	Cotton dressings (non-woven and wax coated cotton dressings)	5 and 10% (w/w)		*this study*
Ex-ovo CAM	n/a	200 μg/day		(Dikici, Mangır, et al., 2019)
	Electrospun polymer nanofibres	250 μg/1 g polymer		
In vivo	Chitosan/collagen hydrogel	1 mg/ml	Promotes angiogenesis and wound healing in healthy rats	(Yar et al., *2017*)
	Alginate Dressing	5 and 10% (w/w)	Promotes angiogenesis and wound healing in diabetic rats	(Azam et al., *2019*)

2dDR solution in ethanol, it resulted in an abrupt release in one day because 2dDR was superficially coated on the wax surface after ethanol was evaporated. In order to facilitate the release of 2dDR from wax, PEG was used in strategies 3, 4 and 6. PEG being polar and soluble in water was added along with 2dDR drug, with the concept it might push 2dDR release from wax by binding itself with 2dDR. As a result, Strategies 3 and 4 resulted in burst release and over 1 day all the 2dDR was released. In strategy 6, a sustained release was observed over 5 days, when 2dDR solution in PEG and liquid paraffin was coated on wax dressing. However, due to this involving two steps, this procedure was not very attractive. In strategy 5, 2dDR release was observed over 5 days, and drug release was stimulated from wax dressings by coating 2dDR direct on wax surface of dressings via dipping wax-coated dressings in 2dDR containing drug carrier solution in liquid paraffin. However, like strategy 6, this was again two steps and not attractive. The best protocol for loading sugar into wax-coated dressings was found to be strategy 7 where we managed to obtain a sustained release of 2dDR over 5 days by following the same procedure of the original industrial recipe without the addition of any extra components such as PEG or ethanol. In this strategy, double the proportion of liquid paraffin was used, which was diluted to give the final coating mixture and hence stimulated the sustained release of 2dDR from dressings. This method of 2dDR loading into the wax-coated dressings showed an average release of 334 μg/day over 5 days.

After the application of a dressing to a wound, following absorption of wound exudate, the dressing may become dried out and develop a rough texture which could disturb the wound area and cause pain to the patient (Dabiri, Damstetter, & Phillips, 2016). Addition of glycerol to the wound dressings may be helpful to avoid this situation by giving softness to the dressings even after these become dried out (Stout & McKessor, 2012). To test the effect of glycerol on moisture retention and sugar release profile in 2dDR loaded cotton wax dressing, the dressings were treated with a 4% aqueous solution of glycerol and left to dry in aseptic conditions. The 2dDR release studies showed glycerol treatment resulted in slightly slower sugar release (over 7 days) compared to untreated 2dDR loaded cotton wax dressings. Thus, glycerol treatment of dressings can also be considered because of the softness it brings to these dressings.

The *in vitro* cytotoxicity assay results demonstrated that there were no adverse effects of these dressings on fibroblast survival. Indeed, the presence of the 2dDR stimulated the metabolic activity of these cells over 7 days. Similarly, we have recently reported that the treatment of 2dDR increases the metabolic activity of cells in a dose-dependent manner (Dikici, Aldemir Dikici, et al., 2020).

The sterilisation of the dressings was confirmed for two reasons. It is essential for their future clinical translation, and it is necessary to prevent contamination of the chick embryos. If a standard method of sterilisation such as gamma radiation had either affected the release of the sugar from the dressing or its biological activity, then this would have been a major problem in the development of the dressing for future clinical use. Hence, our desire to check this out early. Luckily, standard gamma radiation did not affect sugar release or the biological activity of the sugar-loaded dressings in our previous studies in which alginate was used (Azam et al., 2019), and now, in this study, we have confirmed that gamma sterilisation can be effectively used with cotton dressings.

The second point is that the CAM assay is a very good bioassay to evaluate the angiogenic properties of the biomaterials, but the living chick embryo is highly sensitive to infection. Thus, the sterility of the material to be implanted on CAM and the cleanness of the equipment used are critically important not only for the survival rate of the embryos but also for the reliability of the results (Dikici, Claeyssens, & MacNeil, 2019; Dikici, Mangır, et al., 2019; Mangir et al., 2019).

The inclusion of 2dDR in both 150 g/m^2 cotton dressings and cotton wax dressings stimulated angiogenesis either when assessed macroscopically (quantified using photographs of the CAMs) or histologically (quantified based on the histological sections of the CAMs). The histological analysis of the CAMs showed increased blood vessel formation adjacent to the area where the dressings were implanted. The macroscopic evaluation of the CAMs also showed that there were more blood vessels in the 5% 2dDR loaded 150 g/m^2 cotton dressing when compared to controls. Figure 6A- 6B show the connective tissue bed having various cells, including inflammatory cells and blood cells. In Fig. 6 Ac inflammatory cells can be seen along with red blood cells inside the blood vessel. The presence of inflammatory cells in blood vessels of CAM during angiogenesis has previously been reported (Bessa et al., 2015). Inflammatory cells are essential activators of neoangiogenesis via production of cytokines, vascular endothelial growth factor, interleukins (IL-1, IL-2, and IL-8) and platelet activator factor. All of these are cell-specific growth factors, and they play vital roles in the initiation of the inflammatory response, which ultimately leads to neovascularisation and growth of pre-existing capillaries in the CAM (Zijlstra et al., 2006).

Our findings clearly showed the angiogenic potential of both 2dDR loaded cotton dressing and cotton wax dressings where a significantly higher angiogenic response was observed for 2dDR loaded 150 g/m2 cotton dressings. Despite this difference, the main finding is that both dressings are effective at stimulating angiogenesis.

5 | CONCLUSIONS

In conclusion, the introduction of 2dDR into non-woven cotton wound dressings and wax-coated cotton dressings achieved good angiogenic potential in a well-accepted angiogenic bioassay. This is in line with our previous studies in which we have used various biomaterials for the delivery of 2dDR to promote angiogenesis. 5% 2dDR loaded 150 g/m^2 cotton dressing and 5% 2dDR loaded cotton wax dressings showed similar sugar release kinetics over 5 days. Thus, one could choose either dressing for clinical development depending on the wound type. The former dressing would be ideal for treating chronic wounds and ulcers because of its good absorption capacity, while the 2dDR containing cotton wax dressing would be preferable for treating burn wounds because of its non-adhesive properties. Both dressings are based on cotton fibre dressings manufactured in

Pakistan and supplied to various hospitals throughout Pakistan. Thus, there is potential to develop these as effective advanced wound dressings that could be developed for clinical use in Pakistan and indeed in any countries where access to low-cost pro-angiogenic dressings would be beneficial.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTIONS

The study was designed by Muhammad Yar and Sheila MacNeil who analysed the data and played a major role in writing up the manuscript Cotton Craft sponsored the funding of Anisa Andleeb who performed the majority of the research experiments and provided dressings and wax mixtures for the study. Shahid Akhter from Cotton Craft also provided assistance in the formulation of wound dressings based on his industrial experience, Aqif Anwar Chaudhry provided assistance in raising funds for this research work and project management.

Serkan Dikici contributed to writing up the paper, and generating/improving the figures and table, Tayyaba Sher Waris performed cytotoxicity experiments, Muhammad Mustehsan Bashir provided a clinical perspective on the need for pro-angiogenic dressings.

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