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Mammalian Goat Blood Model: An Emerging *In-vitro* Technique for Testing New Compounds for Toxicity

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ABSTRACT

This study was undertaken to investigate the use of mammalian Goat blood as a rapid model for toxicity testing. Four (4) new materials (Niprigel-CP®, Niprigel-K®, Niprigel-A®, Nipricel-C®) and a known polymer, Avicel® pH101 were evaluated. The polymers were incorporated into erythrocytes from goat blood that had been washed with phosphate buffer solution (PBS) and incubated at 37 °C for 2 h. Absorbance of the incubated samples were taken at 415 nm. Percent haemolysis of the new materials compared to that of TritonX 100, was taken as measure of cytotoxicity. Results show that Niprigel-CP® at 0.01 %w/v, induced 411 % haemolysis, while Niprigel-K® (1.0 %w/v) induced 90 % haemolysis. Niprigel-A® and Nipricel-C® were not toxic at all the concentrations investigated, their hemolytic activities were also found to be comparable to that of PBS and Avicel® pH101. The method was found to be a simple, cheap and less time-consuming means of testing for safety and could be an alternative method of safety profiling especially in resource-starved nations.

Keywords: Goat blood, biomaterials, cytotoxicity, *in-vitro* testing

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INTRODUCTION

Safety of substances intended for animal or human consumption whether from chemical or natural origin are paramount to protect the health of the public and the environment. More so, veterinary products are also tested to ensure safe use of products for both household pets and livestock and evidence of safety is a strict regulatory requirement for registration and sale of drugs, cosmetics, food and water.

Testing is a compulsory requirement by regulatory authorities and sometimes, specific methods are recommended for comprehensive evaluation before release to the market. Other times, it is required that, testing of environmental samples is undertaken using different approaches and methods in order to evaluate complex materials and the possible effects such substances may exhibit. The effect maybe different from the behavior of the individual substances of these materials. Herbal medicines are examples of these complex materials because they contain many chemical constituents with specific individual but collective bioactivity including biotoxicity.

Some scholars have opined strongly that, ascertaining the safety and biocompatibility of candidate biomaterials is a crucial aspect of formulation development [1]. Denta *et al* [2] emphasizes this and the need to test for toxicity without the use of animal models. Their study explored novel techniques employed in assessing the risk of cosmetic ingredients where next generation risk assessment (NGRA) approaches such as *in vitro, in silico* or *in chemico* methods are used. Patlewicz *et al* [3] also documented the NRC's (National Research Council) recommendation to consider the use of *in vitro* assay techniques in the determination of toxicity or adverse biological responses. Their study which was aimed at developing ways through which commercially used chemicals can be tested for toxicity also reported that commonly and extensively used *in vivo* methods for toxicity testing based on toxicological responses in animals are time and resource wasting. Hence, they suggested the use of alternative toxicity testing approaches such as toxigenicity, high throughput screening assays, quantitative structure activity relationship, dose-dependent physiological alterations, e.t.c. [3-4]. Various *in vitro* techniques have been developed to this effect but most of the developed and standardized methods for biocompatibility testing involve the use of animal models and collection of blood samples from human subjects which may be expensive, tedious and time consuming [5]. For instance, the use of the mutagen sensitivity assay in a glioma case control study [6] which is based on the assumption that exposure to the neurocarcinogen; acrylamide can cause cellular changes [7-8] (lower DNA repair capacity) in patients with glioma. The

study involved among others the collection of blood from human subjects, isolation, freezing and storing of lymphocytes which could be time and resource demanding. The procedure also reported a major drawback of cell line failure.

Majeed *et al* [9] sacrificed rodents in a bid to assess the safety profile of 40 % garcinol extracted from *Garcinia indica* over a period of 28 days. Similarly, in a study to assess the effect of fipronil insecticides in the Caspian kutum fish, about a hundred fishes were used. The study lasted for 14 days after which alterations in organs were assessed and used as an indication for toxicity [10]. In both cases, the presence or absence of toxicity could have been proven without the use of animal models at the availability of a feasible, cheaper and faster approach.

The use of toll-like receptor (TLR) transfected cell lines to detect icodextrin contamination with bacteria, peptidoglycan and lipopolysaccharides was carried out by Hacine-Gherbia *et al* [11]. Their reports showed the difficulty experienced with the use of cellular models such as monocyte activation test which uses the release of cytokines as a biomarker. The TLR bioassay although sensitive is tedious and lengthy and not practicable for researchers in developing or low-income countries due to cost ineffectiveness.

While it is noted that medicines must be examined in human beings according to strict ethical rules, there is strict prohibition worldwide on giving any products to humans without first testing them in appropriate model systems. Unfortunately, many chemicals in commonly used products such as; pesticides, and other chemicals used in the production of toys, clothing, building materials and furniture, cannot be tested directly in humans without the use of costly models. There is therefore the need to develop and employ easier approaches to determining biocompatibility. Bio-incompatibility can be detected by cytotoxicity which refers to the toxicological risks caused by a material to cells [12] or a measure of specific induced-cell damage. This study seeks to develop a rapid, non-invasive, sensitive, safer and inexpensive *in-vitro* means of determining if an agent has significant amount of biologically harmful extractable. This is in line with the requirement that, a novel toxicity testing method should; minimize the use of animal models, be time and cost

effective, applicable to testing a wide range of existing /new materials and have a strong scientific criteria by which toxicity or risk is evaluated [13].

The method reported herein capitalizes on the principle of hemolysis defined as the destruction of erythrocytes, leading to the release of hemoglobin [14]. Hemolysis testing can be used to determine the biological reactivity of cells following contact with a biomaterial by measuring the free hemoglobin released as a result of cell lysis [15]. Interactions of biomaterials and their components with cellular components may be responsible for toxic tissue reactions, such as inflammation [16], lysis [17], necrosis [18], immunological alterations [19], genotoxicity [20] and apoptosis [18, 21]. Necrosis could occur as a result of oxidative stress of free radicals generated from cellular interactions with biomaterials [22]. Necrotizing cells usually exhibit any of these four basic characteristics; rapid edema, decreased or lost membrane integrity, metabolism shut down and lysis [23] which is the basis of hemolysis assay. This study also provides insight into concentration-toxicity relationship which is necessary to optimize the cytotoxicity and biocompatibility of polymeric drug delivery systems.

MATERIALS/METHOD

Materials

Goat blood, Trisodium citrate (BDH, chemicals Ltd. Poole, England), Phosphate buffer solution (BDH, chemicals Ltd. Poole, England), Tween 80 (Riedel-deHaen, Germany), Polymers (Niprigel-CP®, Niprigel-K®, Niprigel-A®, Nipricel-C® developed at National Institute for Pharmaceutical Development and Research; NIPRD) and Avicel® pH101 (Fluka Biochemika).

Method

The method of Nair et al., [24] was adopted with some modifications. Blood sample from goat was collected in an appropriate vessel containing 4 % trisodium citrate and 10 mL of the blood was centrifuged at 3500 rpm for 5 min. The plasma was discarded and the pellet washed three times with phosphate buffer solution (PBS) pH 7.4 by centrifuging at 3500 rpm for 5 min. Suspension of the blood cells in PBS (3 %) was prepared and 0.5 mL of the suspension was mixed with 0.5 mL of the polymer samples (0.01, 0.03, 0.05 and 0.10 % in PBS). The mixtures were

incubated for 2 h at 37 ºC and centrifuged at 3500 rpm for 10 min. The supernatant was diluted by adding 2.8 mL of PBS to 0.2 mL of the supernatant. The free hemoglobin in the supernatant was measured using the UV-Vis spectrophotometer at 415 nm. Phosphate buffer solution (PBS) and Triton X 100 were used as negative and positive controls respectively. Hemolysis (%) was calculated using the formula:

$$H\ (\%) = \frac{A_t - A_n}{A_p - A_n} \times 100 \ldots..(1)$$

Where:

H = hemolysis,

A_n = absorbance of negative control (PBS)

A_t = absorbance of test sample

A_p = absorbance of positive control (Triton X 100)

RESULTS AND DISCUSSION

Testing for toxicity is essential so as to determine or predict the biological reactivity of the mammalian cells following contact with a substance, extract, chemical, drug or even excipient used as drug carriers in formulations [25]. Polymers have been extensively used as biomaterials; which are constituents of medical devices and drug-delivery systems [24-26]. In drug delivery, both natural and synthetic polymers have found great applicability in promoting targeted drug delivery, achieving controlled or sustained drug release, masking unpleasant drug taste, improvement of drug stability, solubility and bioavailability, e.t.c. [27-28]. These enormous benefits could be undermined if biopolymers pose harm to living tissues. Four (4) new materials (Niprigel-CP®, Niprigel-K®, Niprigel-A®, Nipricel-C®)) and a known standard reference polymer, Avicel® pH101 were evaluated using this model. Niprigel-CP® displayed a concentration dependent increase in cytotoxicity; 0.1 % > 0.05 % > 0.03 % > 0.01 %; the degree of hemolysis observed with this polymer was higher than observed with other polymers at the corresponding concentrations including the standard polymer, Avicel. Hemolysis (%) induced by Niprigel-CP® between 0.03 and 0.1 % was found to be greater than that of the known hemolytic agent, Triton X100. The lowest concentration (0.01 %) on the other hand was found to cause appreciable hemolysis which was about 60 % higher than was observed with the known non-hemolytic agent, PBS. It is

safe therefore to conclude that, Niprigel-CP® is extremely cytotoxic. Figure 1 shows that Niprigel-K®, Niprigel-A®, Nipricel-C® and Avicel pH101 exhibited concentration independent hemolytic activity. Although at the highest concentration (0.1 %), Niprigel-K® was observed to induce higher hemolysis than Triton X100, which shows that it is unsafe at this concentration. Other polymer showed hemolysis (%) less than that observed with Triton X100 and some comparable to that of PBS at all the concentrations investigated. This shows their compatibility with blood cells and suggests that they could be safe.

The trend of safety of these polymers are Niprigel-C® > Niprigel-A® > Niprigel-K® > Niprigel-Cp®. This suggests that Nipricel-C® and Niprigel-A® could be considered safe as biopolymeric drug carriers. Niprigel-k® however, could be employed at concentrations below 0.1 % while Niprigel-Cp® could be presumed to be unsafe.

Figure 2 is a pictorial showing the erythrocyte suspension with and without hemolysis respectively. The dark red color observed where hemolysis occurred is attributed to the presence of hemoglobin [12] leached out of the cells upon exposure to the test polymers.

CONCLUSION

The hemolytic activity of four new biopolymers were evaluated using a new *in vitro* approach. The method proved to be rapid, inexpensive, safe and could be used in predicting the suitability or otherwise of a new biomolecule or excipient intended for drug delivery.

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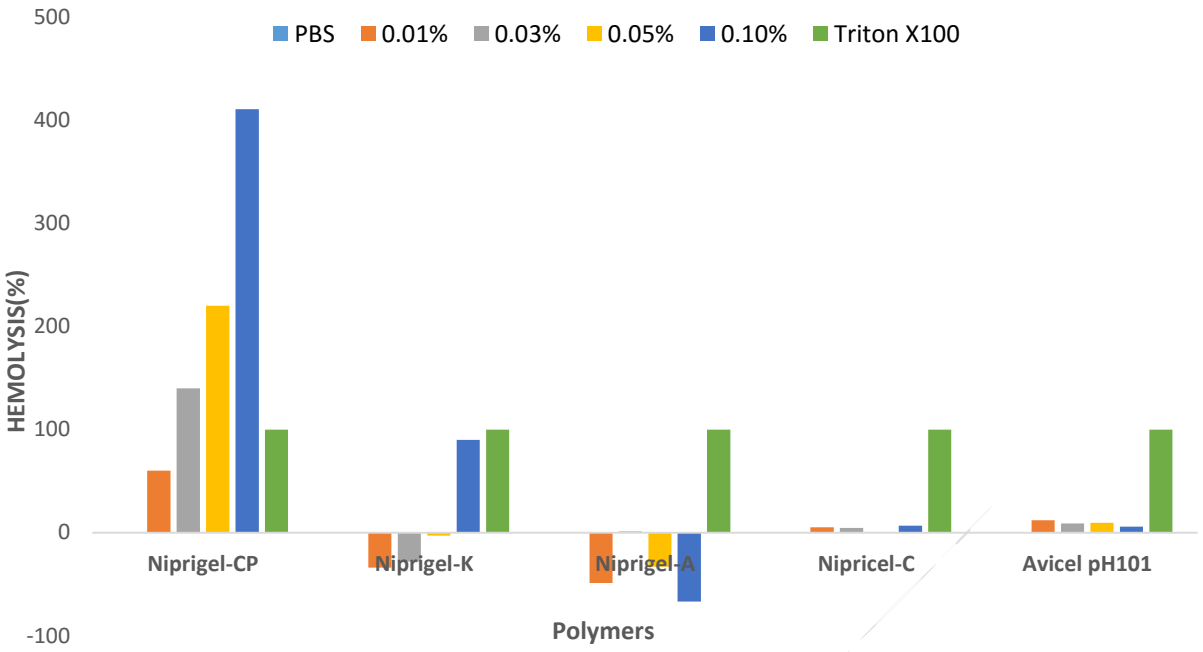

Figure 1: Haemolytic activity of Niprigel-C, Niprigel-A®, Niprigel-K®, Niprigel-Cp® and Avicel

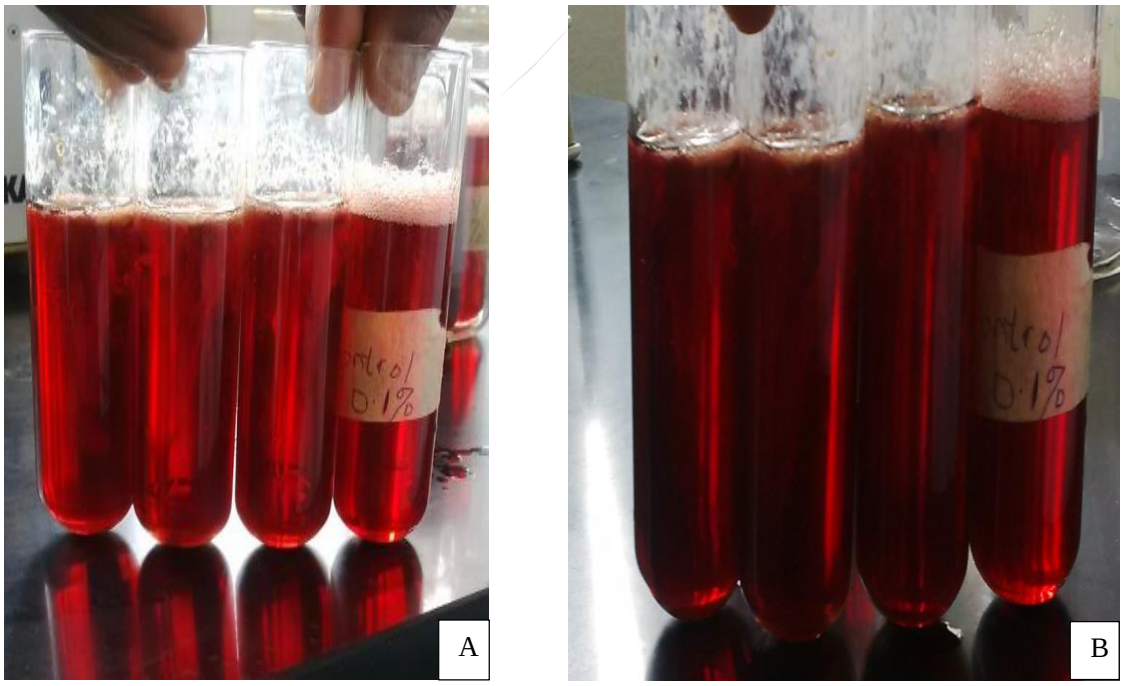

Figure 2: Test tubes containing goat blood + test polymers before (A) and after (B) incubation

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