Followed by a progressive decrease in the glucose level. In rat plasma, a gradual decrease in glucose level was observed up to 32h (139 mg/dl) for free DDA, whereas nano-DDA exhibited a major decrease in glucose concentration at 32h (115 mg/dl) which continued even after 48h (117 mg/dl).

**Conclusion:** DDA prepared using polyvinyl alcohol as a surfactant remained non-toxic till 10µM. OGTT studies revealed an initial increase of glucose at 30 min of DDA which resulted in decreasing the concentration of glucose which could be due to the pronounced antihyperglycemic activity of nano-DDA.

Among the two types of diabetes, Type II Diabetes Mellitus is the most common form of diabetes mellitus among 90-95% of the diabetic population [4], characterized by insulin resistance (IR), which is a hallmark of type 2 diabetes. Impaired insulin secretion and insulin resistance leads to the dysregulation of glucose homeostasis.

According to patient’s perspective, oral administration of the drug is absolutely not painful and omits uncomfortable interventions. However, the oral bioavailability of bioactive compounds is limited by the protease enzymes in the gastrointestinal tract and hydrophobicity of the bioactive compounds [5]. In the field and pharmaceutical industries, the controlled release systems have emerged extensively to release the active substances E.g: drugs and nutrients [6]. The advancement in material science and nanoeengineering are being prioritized for encapsulating therapeutic agents into a nanof ormulation for targeted drug delivery [7, 8].

The pharmacological potential of bioactive compounds enormously depends on their solubility in water [9]. Unfortunately, the sparing solubility in water limits their bioavailability and clinical application. Hence, site-specific or targeted drug delivery, reduction of systemic toxicity, improvement of physicochemical property, protection from biochemical degradation and improvement of environmental stability are the major challenges for designing an ideal delivery system [2]. Recently, polymer-based drug delivery systems ventured many avenues for increasing efficacy and specific targeting of new, as well as already existing drugs. In this perspective, the biodegradable polymers are of prime interest due to their advantage of elimination of polymer metabolites out of the body by innate metabolic processes [10].

14-deoxy-11, 12-didehydroandrographolide was used in the present study as a demiphenoid of the annual herbaceous plant Andrographis paniculata (Family: Acanthaceae) which have been used for many ailments in India as well as in many Asian countries [11]. The limited availability of pharmacokinetic data of DDA resulted in the delay of further drug development and clinical uses. In our previous study, the enhanced bioavailability of nanoencapsulated DDA in experimental diabetic rats was reported [12]. In this present study, nano-DDA was formulated with two types of surfactants and their cell viability was analysed and the aim is to study the augmented glucose tolerance and release efficacy of the nanoencapsulated DDA (nano-DDA).

**MATERIALS AND METHODS**

**Chemicals and reagents**

Streptozotocin, Nicotinamide, Metformin, Polyoxyvinyl alcohol and Tween 20 were procured from Himedia, Mumbai. Polycaprolactone was purchased from Sigma-Aldrich limited, Bangalore. 14-deoxy, 11, 12-didehydroandrographolide was purchased from Apeksha Research centre private limited, Indore, Madhya Pradesh, India.

**Cell culture**

Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) was used to culture L6 myoblasts (rat skeletal muscle cells) with streptomycin (75 µg/ml), penicillin (120 units/ml), amphotericin B (3 µg/ml) and gentamycin (160 µg/ml) in 5% CO₂.
Formulation and characterization of nanoencapsulated DDA

Four formulations were attempted by varying the drug to polymer ratio, type of surfactant and sonication time. Polycaprolactone (PCL) was used as a biodegradable polymer. The nanoparticles containing PCL-DDA conjugate and free PCL were fabricated by solvent evaporation technique [13]. Briefly, known amounts of the DDA and PCL were added to 0.5 ml of dichloromethane, stirred to ensure that the materials were dissolved. The organic phase was introduced slowly into either 0.5% PVA or 1% Tween 20 solution resulting in an emulsion. Bandelin sonopuls sonicator (model UW2070, BANDELIN electronic GmbH and Co. KG, Berlin) was used to break down the emulsion. Bandelin sonopuls sonicator (model UW2070, BANDELIN electronic GmbH and Co. KG, Berlin) was used to break down the emulsion. PCL was added to 0.5 ml of dichloromethane, stirred to ensure that the materials were dissolved. The organic phase was introduced slowly into either 0.5% PVA or 1% Tween 20 solution resulting in an emulsion.

Assessment of cytotoxicity by MTT assay

The cytotoxic effect of nanoencapsulated drug prepared with polyvinyl alcohol (PVA) and tween 20 as surfactants was analyzed by MTT [3-14, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT)] assay. The MTT assay is a colorimetric assay for evaluating cell viability. NAD(P)H-dependent cellular oxidoreductase enzyme reduces the yellow tetrazolium dye (MTT) to insoluble formazan crystals (purple colour). These enzymes are present largely in the cytosolic compartment of the cell. A solubilization solution was added to dissolve the insoluble product. The absorbance was read at 570 nm by a UV spectrophotometer [12-16].

Measurement of release efficacy

After one week washout period, experimental animals were administered with free DDA (50 mg/kg b.w.) and nano-DDA (50 mg of nanoparticle-containing 9.4 mg DDA/kg b.w.).

The area under the curve (AUC) was calculated using the trapezoidal method. The release efficacy of DDA from polymer matrix formulation was extrapolated using the AUC. Release efficacy percentage was calculated using the following formula [17]:

Release efficacy percentage = \( \frac{AUC}{AUC_0} \times 100 \)

Where AUC is the area under the curve of drug release chromatogram and AUC0, is the area under the curve of the standard sample.

Experimental animals and diet

Healthy adult male Wistar rats (150-200g) were used for the study. Rats were housed in polypropylene cages lined with husk in standard environmental conditions (temperature 25±2 °C with dark/light cycle 12/12h; 55±10% humidity). Priorly the experimental protocol was approved by the Institutional Animal Ethical Committee and was approved by the same before beginning the experiment [53/IAEC/2011]. Animals were fed with standard pellet diet and water ad libitum.

Experimental induction of diabetes

Streptozotocin (STZ) and nicotinamide (NIC) were dissolved in freshly prepared 0.1M citrate buffer (pH 4.5) and in normal physiological saline respectively and stored on ice prior to use. A single injection of Streptozotocin (45 mg/kg body weight), 15 min after the administration of nicotinamide (110 mg/kg body weight) was given intraperitoneally to induce Diabetes mellitus in overnight fasted rats. Hyperglycemia was confirmed by the raised blood glucose levels, determined on 3rd and then on 7th day after induction. The rats with blood glucose levels above 250 mg/dl were used for the study [18].

Experimental design and treatment of animals

Experimental design of free DDA

The rats were divided into seven groups comprising of five rats in each group. All rats except control and the diabetic group were administered with the drug (orally) for 45 d.

Group I–Control rats received Carboxymethylcellulose (0.5%)

Group II–Control rats administered orally with DDA (50 mg/kg body weight)

Group III–Diabetic rats

Group IV–Diabetic rats administered orally with DDA (10 mg/kg body weight)

Group V–Diabetic rats administered orally with DDA (25 mg/kg body weight)

Group VI–Diabetic rats administered orally with DDA (50 mg/kg body weight)

Group VII–Diabetic rats administered orally with Metformin (500 mg/kg body weight)

Experimental design for nano-DDA

Group I–Control rats received Carboxymethylcellulose

Group II–Control rats administered with nano-DDA (50 mg/kg b.w.)

Group III–Diabetic control rats (NIC+STZ induced)

Group IV–Diabetic rats administered with DDA (50 mg/kg b.w.)

Group V–Diabetic rats administered with nano-DDA (50 mg/kg b.w.)

Group VI–Diabetic rats administered with nano-DDA (25 mg/kg b.w.)

Group VII–Diabetic rats administered with Metformin (500 mg/kg b.w.)

Animals were administered with DDA for 45 d (1 ml/rat) in 0.5% carboxymethyl cellulose as a vehicle. DDA and Metformin were suspended in 0.5% CMC prepared in autoclaved distilled water prior to oral administration to experimental animals.

Oral glucose tolerance test (OGTT)

After treatment for 45 d with free and nano-DDA, glucose tolerance test was performed on overnight fasted rats [19]. Animals of all groups were administered with glucose (2g/kg) orally. Group IV, V, VI were treated orally with drug and group VII was administered with metformin (500 mg/kg), 30 min prior to oral glucose administration. The blood samples were withdrawn through retro-orbital plexus at 30, 60, 90 and 120 min after glucose administration.

Concentration of glucose

Blood samples were collected from retro-orbital plexus of the rats at predetermined time intervals and plasma was separated immediately by centrifuging of heparinized blood at 3500 rpm for 10 min. The concentration of glucose in plasma samples was estimated using Robonik priestet GOD-POD glucose estimation kit, Robonik (India) Private limited, Mumbai, India.

Statistical analysis

Results were expressed as mean±SD. The data were analysed using Graphpad Prism 5.03 statistical software (Graphad software Inc., La Jolla, CA). The statistical significance was evaluated by one-way analysis of variance (ANOVA) and Dunnett’s multiple comparison tests was performed to determine significant differences between groups. The criteria for statistical significance were P<0.05.

RESULTS AND DISCUSSION

Cell viability assay

The capability of cells in reducing MTT delivers an indication of mitochondrial integrity and activity, which could be inferred as a measure of viability of cell [20]. The results of the cell viability study were presented in the form of percentage viability remains after treatment with PVA and Tween 20 formulations (fig. 1). The results revealed that the nano-DDA (Tween20) treated cells, exhibited a significant, (P<0.05) decrease in cell viability on the L6 myoblasts. The percentage viability of L6 cells after treatment with the aforementioned formulation was found to be 67% in 1nM. A major decline in the cell viability was noticed at 10µM (34%). But the cells treated with nano-DDA (PVA) did not display any change in cell viability.
viability (98% viable on par with the control). In addition, nano-DDA (PVA) incubated L6 myoblasts did not elicit cytotoxicity to L6 myoblasts up to 10µM. This is suggestive of the compatibility of nano-DDA (PVA) with a living system. Hence nano-DDA with PVA as the surfactant has been used in further experimental procedures, omitting nano-DDA with tween20 as surfactant since it elicited a decline in cell viability. Chibueze Ihekwereme (2014) reported that calu-3 cells (bronchial adenocarcinoma of the airway) incubated with 0.2% Tween 20 for 60 min exhibited cytotoxicity with 57.1%±8.1% viability of the cells [21]. Atefeh Hekmat et al. (2016) stated that Docetaxel-Loaded Nanomicelles using Tween 20 as a surfactant resulted in a higher C26 cell death rate [22]. Vimala et al. (2011) reported that usually at the end of the preparation of nanoencapsulation, the surfactant used was removed by centrifuging the nanoparticles. But the surfactant which adsorbed on the surface of the nanoparticles could not be removed completely due to the formation of the interconnected network formed on the surface [23]. According to Sahoo et al. (2002) up to 13% of surfactant level is permissible which could influence the physical and biological properties of the nanoformulation [24].

Release efficacy percentage (%)

The in vitro release of DDA loaded nanoparticles appeared to be two-stage processes. Initially there was a burst release of DDA from polymer matrix was observed. After that release of DDA was at constant rate. Generally, drug release from the polymer matrix depends on the diffusion of the drug, the erosion and the swelling of the polymer matrix and degradation of polymer [25]. The release efficacy of DDA from nanoparticulate matrix is tabulated (table 1). Release of DDA from nano DDA (DDA loaded PCL nanoparticles) occurred in a controlled manner. When the nanoparticles were exposed to the aqueous environment, the initial burst release which was less than 20% that lasted up to 4h. Later, a gradual and sustained release of drug from the nanoparticulate matrix, which increased up to 74% in 16h followed by a fall in release efficacy, was observed. At 48 h about 4% of DDA release was observed. The slower and sustained release of DDA may possibly be endorsed the diffusion of the DDA loaded within the core of the nanoparticle matrix. You ling et al. (2008) reported that paclitaxel loaded PLGA nanoparticles exhibited a two-staged release mode from the nanoparticle [25].

Table 1: Release efficacy of DDA from PCL polymer matrix

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>AUC (nano-DDA) (ng)</th>
<th>Release efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>15.59±0.72</td>
<td>1.20±0.03</td>
</tr>
<tr>
<td>2</td>
<td>91.76±2.45</td>
<td>7.09±0.96</td>
</tr>
<tr>
<td>4</td>
<td>253.41±4.67</td>
<td>19.59±7.5</td>
</tr>
<tr>
<td>8</td>
<td>572.02±6.67</td>
<td>44.22±2.49</td>
</tr>
<tr>
<td>16</td>
<td>961.72±8.89</td>
<td>74.35±2.96</td>
</tr>
<tr>
<td>24</td>
<td>499.66±3.26</td>
<td>38.63±2.56</td>
</tr>
<tr>
<td>32</td>
<td>182.16±2.95</td>
<td>14.03±0.83</td>
</tr>
<tr>
<td>48</td>
<td>54.82±2.56</td>
<td>4.23±0.025</td>
</tr>
</tbody>
</table>

The values are expressed as mean±SD, n=3 consecutive experiments, AUC-area under curve

Oral glucose tolerance test

The effect of DDA on reducing the plasma glucose level in glucose loaded (2g/kg) rats was displayed in fig. 2. Initially, there was a sharp elevation in 30 min after glucose loading in all groups. DDA treated rats displayed a dose-dependent decline in the glucose level from 60 to 120 min similar to the control rats. However, the restoration from hyperglycemia was not observed even at 120 min in the diabetic rats, which differs significantly (P<0.05) with control rats, in which the glucose level gradually reverted back to the normal level after 30 min. The results from the observation clearly indicated that the increment in the blood glucose found in the oral glucose tolerance turned into significantly greater in diabetic rats than the treatment groups. Zhaoxia Liu et al. (2013) reported that compared to diabetic control, DTH extract treated group showed significant hypoglycemia and steady decline which exhibited a similar effect to metformin on OGGT [26]. Gerardo et al. (2000) reported that insulin-loaded PLGA formulation did not show any increase in blood glucose levels even at 800 mg/kg subcutaneous load of glucose to rats, which substantiated that the formulation is bioactive and useful in controlling elevated blood glucose levels [27]. Lei song et al. (2014), stated that the blood glucose was found to be effectively lowered in overnight fasted diabetic animals administered with nanoeencapsulated insulin-chitosan complexes [28].
Fig. 2: Effect of free and nano-DDA on Oral glucose tolerance test in streptozotocin-nicotinamide-induced diabetic rats. Data represented as mean±SD, n=5.*p<0.05 as compared with diabetic group, #p<0.05 as compared with control, dia-diabetic

Concentration of glucose

The blood plasma profile of the rats after oral administration of free and nano-DDA was analysed. As displayed in table 2, the plasma of free DDA treated rats exhibited a dose-dependent decrease in glucose concentration. A major decrease in glucose level (115 mg/dl) was observed at 2h in which the T_max for free DDA as reported by Nagalakshmi et al (2017) [12]. There was a gradual decrease in glucose level which lasted up to 32h. At 48 h, an elevation in the glucose level was observed which could be due to the lesser concentration of DDA in plasma. On the other hand, the nano-DDA treated rats showed a dose dependent decrease in the glucose concentration and a major decrease in the glucose level (102 mg/dl) at 16h, which was the T_max for nano-DDA as reported in our previous studies. In contrast to free DDA, nano-DDA maintained a declined glucose level of 117 mg/dl even at 48 h, which was about 189 mg/dl in free DDA treated rats at 48h. As reported by Zadeh et al. (2017), nanoencapsulation of insulin using PVA as a surfactant enhanced the absorption of insulin via the intranasal route of delivery [17]. Seema et al. (2017) reported that upon administration of repaglinide loaded microspheres to experimental diabetic rats, a glucose level of the formulation started to decrease significantly after 1 h which continued up to the eighth h indicating controlled release of drug from the formulations [29].

Table 2: Glucose concentration (mg/dl) at various time points after oral administration of nano-DDA (PVA) formulation to diabetic rats

<table>
<thead>
<tr>
<th>Time(h)</th>
<th>free DDA</th>
<th>nano-DDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>14±2.4</td>
<td>12±2.78</td>
</tr>
<tr>
<td>1</td>
<td>13±1.5</td>
<td>12±2.67</td>
</tr>
<tr>
<td>2</td>
<td>11±1.37</td>
<td>11±2.89</td>
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<td>4</td>
<td>12±1.37</td>
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<tr>
<td>8</td>
<td>12±2.65</td>
<td>10±2.82</td>
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<tr>
<td>16</td>
<td>12±2.33</td>
<td>10±2.98</td>
</tr>
<tr>
<td>24</td>
<td>13±2.9</td>
<td>10±2.95</td>
</tr>
<tr>
<td>32</td>
<td>13±2.48</td>
<td>11±3.1</td>
</tr>
<tr>
<td>48</td>
<td>18±3</td>
<td>11±2.7</td>
</tr>
</tbody>
</table>

Data represented as mean±SD, n=5, PVA-polyvinyl alcohol

CONCLUSION

The oral administration of nanoencapsulated DDA exhibited an enhanced oral bioavailability of DDA than free DDA, by reducing the first-pass metabolism of DDA. However, further studies using clinical trials may confirm the results obtained in this study. Henceforth, the nano-DDA can be considered as a potential therapeutic candidate in the antidiabetic drug. This work opens a door for discovery of novel potential antihyperglycemic agents.

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CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

REFERENCES


