Introduction

The degree of damage to the intestinal mucosa of laboratory animals is commonly evaluated in research projects aimed at the diagnosis and follow-up of intestinal integrity, especially in studies that involve experimentally-induced gastrointestinal diseases or in the development of potential therapeutic agents. The assessment of intestinal mucosal integrity is typically based on the interpretation of tissue specimens collected surgically, endoscopically or at necropsy. However, this approach has shortcomings from scientific and animal welfare perspectives. Histological samples may be incorrectly interpreted, and they only provide morphological insights rather than information on intestinal wall permeability. Furthermore, an intestinal biopsy is an invasive procedure requiring anaesthesia, and additional animals serv-
ing as controls must be used (Kim & Berstad, 1992; Hall, 1994; Ahn et al., 2001; Hoffmann et al., 2002; Jurjus et al., 2004; Chen et al. 2007). IP tests, however, allow the non-invasive assessment of intestinal mucosal integrity. These tests have been successfully applied in humans and a variety of animal species to detect primary intestinal permeability defects, and also to monitor recovery from intestinal damage after therapy in both clinical and research settings (Bjarnason et al., 1995; Hollander, 1999; Hall, 1999).

IP test protocols have frequently involved the use of $^{51}$chromium-labelled ethylenediamine tetra-acetic acid ($^{51}$Cr-EDTA) and a variety of sugars (e.g. lactulose and rhamnose) as probe markers. However, disadvantages associated with the use of $^{51}$Cr-EDTA radioactivity and bacterial degradation of the saccharides has led to the search for better probe candidates. More recently, iohexol, an iodinated contrast agent commonly used in medical imaging, has also been successfully applied as an IP marker for the non-invasive screening of intestinal damage in laboratory rats and humans. The main advantages of using this substance are that it is non-radioactive, biologically inert, widely available in radiological units, relatively inexpensive, and also allows the simultaneous examination of the gastrointestinal tract using other imaging techniques (Stordahl 1988; Bjarnason et al., 1995; Halme et al., 1993; Halme et al., 1997; Halme et al., 2000; Hall, 1999; Andersen et al., 2001; Frias et al., 2009).

Furthermore, IP testing methodology has essentially been standardized in human and veterinary patients, although in laboratory rats there is a substantial lack of uniformity in testing protocols. For example, IP testing has been inconsistently attempted in this species in vivo on either anaesthetized or conscious subjects, after notably variable timed urinary recoveries, from tissue specimens collected from anaesthetized animals via invasive methods, or ex vivo during post-mortem examinations (Bjarnason et al., 1985; Willoughby et al, 1996; Andersen et al., 2001; Milde et al., 2003).

The objective of this study was to establish an improved IP test protocol using iohexol that is able to discriminate between healthy and affected rats, and that is consistent with Russell and Burch’s guiding principles on the reduction and refinement of laboratory animal use.

### Materials and methods

#### Experimental animals, housing and husbandry

Thirty female adult Hsd:Sprague Dawley™ SD™ (SD) rats obtained from a breeding colony kept under semi-barrier conditions at the Central Animal Laboratory, University of Turku, Finland, were used in this study, which was part of another study reported previously (Frias et al., 2009). At the commencement of the study, the rats were 12 weeks old and ranged in body weight from 200 to 250 g. The rats were housed in groups of six, and grouping was decided based on rat availability from the breeding colony. They were maintained in stainless steel cages (59.5 x 38.0 x 20.0 cm) with solid bottoms and Aspen chips as bedding (Tapvei Ltd, Kaavi, Finland), with enrichment consisting of an Iglo and some nesting material. Cage change was undertaken twice a week. The environment in the room consisted of a temperature range of 20 to 23 °C, a relative humidity of 50 to 60%, and artificial illumination with a 12-h light/dark cycle (lights on at 06:00 am). Throughout the study period, all the rats were fed a standard rat chow (SDS, Special Diet Services, Whitham, Essex, UK) ad libitum, and tap water was provided without restrictions in polycarbonate bottles. Prior to the exposure to dextran sulphate sodium (DSS), all the rats were acclimatized for 21 days and were determined to be healthy on the basis of individual physical examinations, and pathogen-free based on the results of routine microbiological screening performed on the colony in accordance with European recommendations (Nicklas et al., 2002).

#### Ethical statement

The rats were cared for and used in accordance with Finnish legislation and Council of Europe Convention ETS 123 on the use of vertebrate animals for scientific purposes (Council of Europe, 1986; Finnish Government, 1985; Finnish Government, 1996), and the experimental protocol was part of a project approved by the Ethics Committee for Animal Experiments of the University of Turku, Finland.

#### Study design

The oral iohexol IP test in urine was carried out twice in each of the thirty SD rats included in this study, once before and then seven days after the experimental induction of gastrointestinal damage.
**Induction of gastrointestinal damage**

Gastrointestinal damage was induced by the seven-day administration of 5% dextran sulphate sodium (DSS) in drinking water, which has been shown to produce symptoms in laboratory rats comparable to the inflammatory bowel disease (IBD) observed in humans (Gaudio et al., 1999; Chen et al., 2007; Frias et al., 2009).

**Oral iohexol IP test measured in urine**

Immediately before the test was carried out, the body weight of the rats was measured. Next, 1 ml of Omnipaque 300° (iohexol, 647.1 mg/mL) was dosed intragastrically to each rat using a feeding tube. No sedative drug was used before, during or after administration. The animals were placed in individual metabolic cages for 24 h for urine collection. After all urine had been recovered, the volumes were recorded and the samples frozen at -18 ºC until later analysis. If oesophageal reflux of iohexol or faecal contamination of urine was observed, the test was cancelled.

**Laboratory analysis of iohexol and creatinine**

Iohexol concentration in urine was analysed by high-performance liquid chromatography with ultraviolet detection ([HPLC]-UV) after solid phase extraction according to a previously published method (Klenner et al., 2007). The formula to calculate the percentage of iohexol excreted in urine is provided in Table 1.

To assess the possible toxic effects of DSS on kidney function, creatinine was determined in all urine samples using a Konelab 30i automatic analyser (Thermo Scientific, Waltham, MA, USA). The iohexol-to-creatinine ratio was calculated similarly to the urinary protein-to-creatinine ratio for the assessment of proteinuria in dogs (White et al., 1984; Grauer et al., 1985).

**Statistical methods**

Statistical analysis was performed using SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). The data were analysed with the Wilcoxon signed-ranks test, and were expressed as the median and interquartile range (IQR).

**Results**

Twenty-eight SD rats were enrolled in the study after the exclusion of two because of oesophageal reflux. Rats exposed to DSS showed evidence of ulcerative colitis based on physical examination and evidence of changes in faecal consistency, diarrhoea and haematochezia. (Gaudio et al., 1999) All serum creatinine concentrations (n = 27) suggested normal renal function.

The median (IQR) percentage (%) of administered iohexol in urine of healthy rats was 0.54% (0.36–0.75%), whereas the respective value after DSS administration was 11.42% (5.58–15.37%). The median (IQR) iohexol/creatinine ratio was 0.05 (0.03–0.06) in healthy rats and 1.38 (0.76–2.49) in rats with IBD.

Figure 1 presents percentile plots of urinary iohexol and the iohexol-creatinine ratio before and after the induction of ulcerative colitis by adding 5% DSS to the drinking water for seven days. Nonparametric comparison of the urinary excretion of iohexol as well as the iohexol/creatinine ratio demonstrated a statistically significant difference (P < 0.001) between healthy rats and those with colitis.

**Discussion**

IP may be assessed by measuring the cumulative urinary excretion of an orally-administered dose of iohexol. An increased IP is reflected by a higher excretion of iohexol in urine due to a higher permeation rate of the probe across the damaged intestinal mucosa of animals with enteric abnormalities. In the present study in laboratory rats, the median 24-h urinary recovery of iohexol after the oral administration of iohexol was 0.54% in healthy rats and 11.42% in rats with ulcerative colitis, indicating significantly higher excretion of the contrast medium in rats with enteropathy. These findings support the use of the present iohexol IP test protocol to detect intestinal alterations in a rat model of IBD. The data are in
agreement with the values reported by other research groups using a different iohexol IP test protocol in rats with experimental enteropathies (Stordahl, 1988; Stordahl, 1988; Laerum et al., 1990; Solheim et al., 1991; Andersen et al., 1992). Our results for iohexol recovery alone are also consistent with the iohexol/creatinine ratios, supporting the conclusion that alterations in the urinary excretion of iohexol were not confounded by possible renal dysfunction due to the DSS in the drinking water, but were only attributable to the higher intestinal permeation of iohexol through the gut mucosa.

Iohexol is a widely used contrast medium in radiographic departments for X-ray diagnostic investigations and kidney function analysis. In addition, iohexol was recently suggested and successfully used as an IP probe in laboratory rats and humans, because this molecule also meets the core physicochemical criteria of an IP probe. However, in contrast to the most commonly used IP markers such as $^{51}$Cr-EDTA and the ratio of lactulose and rhamnose, iohexol is non-radioactive, is not inconsistently degraded by intestinal bacteria, and has more potential applications than other probes, as it may be simultaneously used in the radiographic examination of intestinal morphology by x-ray fluorescence and possibly also computed tomography densitometry (Grönberg et al., 1983; Stordahl, 1989; Andersen et al., 1992; Halme et al., 1993; Halme et al., 1997; Halme et al., 2000; Andersen et al., 2001).

Table 2. Individual results from the iohexol intestinal permeability test before and after administration of DSS for induction of intestinal damage in laboratory SD rats.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Iohexol Before</th>
<th>Iohexol After</th>
<th>Iohexol-creatinine ratio Before</th>
<th>Iohexol-creatinine ratio After</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.83</td>
<td>9.74</td>
<td>0.07</td>
<td>0.72</td>
</tr>
<tr>
<td>2</td>
<td>1.15</td>
<td>5.58</td>
<td>0.08</td>
<td>1.46</td>
</tr>
<tr>
<td>3</td>
<td>0.65</td>
<td>3.2</td>
<td>0.05</td>
<td>0.76</td>
</tr>
<tr>
<td>4</td>
<td>0.19</td>
<td>5.25</td>
<td>0.02</td>
<td>0.88</td>
</tr>
<tr>
<td>5</td>
<td>0.75</td>
<td>2.42</td>
<td>0.06</td>
<td>0.4</td>
</tr>
<tr>
<td>6</td>
<td>0.75</td>
<td>1.84</td>
<td>0.05</td>
<td>0.29</td>
</tr>
<tr>
<td>7</td>
<td>0.59</td>
<td>12.54</td>
<td>0.05</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>0.12</td>
<td>20.02</td>
<td>0.01</td>
<td>2.55</td>
</tr>
<tr>
<td>9</td>
<td>0.36</td>
<td>9.82</td>
<td>0.04</td>
<td>2.77</td>
</tr>
<tr>
<td>10</td>
<td>0.43</td>
<td>17.2</td>
<td>0.04</td>
<td>2.49</td>
</tr>
<tr>
<td>11</td>
<td>0.85</td>
<td>18.77</td>
<td>0.08</td>
<td>2.83</td>
</tr>
<tr>
<td>12</td>
<td>0.81</td>
<td>1.57</td>
<td>0.07</td>
<td>0.48</td>
</tr>
<tr>
<td>13</td>
<td>1.25</td>
<td>3.94</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>14</td>
<td>0.85</td>
<td>15.36</td>
<td>0.08</td>
<td>2.3</td>
</tr>
<tr>
<td>15</td>
<td>0.52</td>
<td>10.7</td>
<td>0.05</td>
<td>2.43</td>
</tr>
<tr>
<td>16</td>
<td>0.44</td>
<td>4.16</td>
<td>0.03</td>
<td>0.41</td>
</tr>
<tr>
<td>17</td>
<td>0.54</td>
<td>15.83</td>
<td>0.06</td>
<td>1.61</td>
</tr>
<tr>
<td>18</td>
<td>0.63</td>
<td>23.71</td>
<td>0.05</td>
<td>2.79</td>
</tr>
<tr>
<td>19</td>
<td>0.31</td>
<td>8.77</td>
<td>0.03</td>
<td>2.67</td>
</tr>
<tr>
<td>20</td>
<td>0.32</td>
<td>8.77</td>
<td>0.02</td>
<td>1.33</td>
</tr>
<tr>
<td>21</td>
<td>0.54</td>
<td>12.05</td>
<td>0.04</td>
<td>1.34</td>
</tr>
<tr>
<td>22</td>
<td>0.37</td>
<td>21.64</td>
<td>0.03</td>
<td>3.04</td>
</tr>
<tr>
<td>23</td>
<td>0.48</td>
<td>10.13</td>
<td>0.04</td>
<td>1.51</td>
</tr>
<tr>
<td>24</td>
<td>0.3</td>
<td>15.37</td>
<td>0.03</td>
<td>1.51</td>
</tr>
<tr>
<td>25</td>
<td>0.3</td>
<td>12.05</td>
<td>0.03</td>
<td>1.24</td>
</tr>
<tr>
<td>26</td>
<td>0.56</td>
<td>12</td>
<td>0.05</td>
<td>1.17</td>
</tr>
<tr>
<td>27</td>
<td>0.81</td>
<td>12.58</td>
<td>0.06</td>
<td>1.38</td>
</tr>
<tr>
<td>28</td>
<td>0.38</td>
<td>11.42</td>
<td>0.03</td>
<td>0.96</td>
</tr>
</tbody>
</table>

* Not determined, n.d.
Intestinal damage may be equivalently evaluated via either histopathological examination or IP testing. However, the latter method is less invasive than the former, since tissue specimens collected surgically, endoscopically or at post-mortem are all obviated using IP tests. IP testing in rats is carried out via the oral administration of a probe such as iohexol and the subsequent measurement of excretion in urine. This means of evaluating intestinal damage may contribute to the guiding principles of reduction and refinement proposed by Russell and Burch in the 1950s (Russell and Burch, 1959), which are currently legal obligations on the use of and care for laboratory animals. IP testing may allow the number of test animals to be reduced because the individuals used in experiments may act as their own controls, and additional control animals are therefore unnecessary. In addition, IP tests allow assessment of the intestinal mucosa in conscious animals without the need for invasive procedures requiring anaesthesia (Stordahl, 1988; Stordahl, 1988; Andersen et al., 2001), and specific observations such as responses of intestinal integrity to novel therapeutics may be followed in the same animals over time. In this way, fewer animals are used and the quality of the scientific data collected is also improved, because intra-individual comparisons more closely resemble the clinical situation.

It is also notable that the more rapid urinary recovery of iohexol may considerably improve the welfare of the rats, as unnecessarily prolonged housing in metabolic cages may be avoided. However, in rats with gastrointestinal disease, a longer collection period may be preferred to increase the test sensitivity. This is because affected animals become dehydrated as a consequence of disease symptomatology, which leads to a reduced urinary output and prevents the rapid acquisition of the minimal volume of urine required for laboratory analysis of iohexol (Klenner et al., 2007).

In summary, the present study supports the use of a refined IP protocol using iohexol for the evaluation of intestinal mucosal damage in laboratory rats. The results reported here indicate that the iohexol IP test performed in this way is able to discriminate between healthy rats and those with gastrointestinal disease. Additionally, compared with some other approaches for assessing intestinal mucosal integrity, this non-invasive test is in closer accordance with the guiding principles of reduction and refinement of laboratory animal use.

Acknowledgements

The research described in this study was conducted in the facilities of the Central Animal Laboratory, University of Turku, Finland. The authors express their gratitude to Laura Lönnberg for performing the solid phase extraction and to Ilkka Saastamoinen and Merja Pöytäkangas for the HPLC-UV determinations. Financial support for this study was obtained from the Academy of Finland. The work was also funded by the European Commission's 5th Framework Programme 'EU&Microfunction': Functional Assessment of Interactions between the Human Gut Microbiota and the Host (QLK1-CT-2001-00135). This article does not necessarily reflect the views of the Commission and in no way anticipates its future policy in this area.

References


Willoughby R, K Harris, M Carson, C Martin, M Troster, G DeRose, W Jamieson, R Potter. Intestinal mucosal permeability to 51Cr-ethylenediaminetetraacetic acid is increased after bilateral lower extremity ischemia-reperfusion in the rat. Surgery 1996,120, 547-553.