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SCIENCE DIRECT
European Journal of Medicinal Chemistry 41 (2006) 605–610
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://france.elsevier.com/direct/ejmech

Original article

Prediction of human absorption of natural compounds by the non-everted rat intestinal sac model

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Received in revised form 20 January 2006; accepted 23 January 2006
Available online 20 March 2006

Abstract

A major concern in natural drug research is that many substances with potent biological activity in vitro are unable to generate good activity in vivo owing to their poor water-solubility, poor permeability and/or poor stability. The permeability of drug candidates across the intestinal mucosa is one of the most important factors in defining drug bioavailability and biological activity. In order to screen promising compounds for further investigation, a non-everted rat intestinal sac model has been developed successfully to assay the permeability of natural compounds and to predict their human absorption. In this system, the drug solution was placed in non-everted intestinal sacs (NEIS), which were placed in an acceptor solution and the permeability of drug across intestine walls was determined. The feasibility of this method has been validated and demonstrated for 11 model compounds chosen from currently marketed drugs whose human fraction absorbed (F_a) data have been reported. The results of the studies indicate that a good relationship exists between the permeability of the model drugs and their corresponding F_a data. The permeability of 13 natural compounds was evaluated using this system. Only fraxinellone and vitexin-7-glucoside exhibited high intestinal permeability, and predictive of excellent human absorption, which awaits confirmation from further investigation in vivo. This model provides an alternative method to everted intestinal sacs for the evaluation of in vitro permeability in rats, and for estimating human absorption of drugs. It may therefore hold great promise for oral absorption screening of new drug candidates.

Keywords: Non-everted intestinal sac model (NEIS); Everted intestinal sacs (EIS); Human fraction absorbed; Permeability; Natural compounds

1. Introduction

With the application of modern isolation technology and high throughput biological screen capability, more and more natural compounds with biological activity are being isolated and identified. However, many of these compounds with potent activity in vitro fail to generate good activity in vivo owing to their poor water-solubility, poor permeability and/or poor stability. The permeability of drug candidates across the intestinal mucosa is one of the most critical factors in defining drug bioavailability and biological activity [1].

A number of in vivo, in situ, and in vitro experimental methods have been developed to determine intestinal drug permeability, such as artificial membranes, cultured cells, isolated tissues, and organ perfusions [2,3]. However, these test systems always represent a compromise either between high throughput with low predictive potential or between low throughput with high predictive potential [4]. For the large number of synthetic drug candidates generated by combinatorial chemistry and high throughput screening, a higher throughput method for evaluating intestinal permeability is highly desired. In contrast, for the precious and relatively small number of natural compounds isolated from plants or animals, a low throughput approach with high predictive potential is believed to be more practical.

The rat was shown to be suitable to predict human absorption from pre-clinical oral absorption studies as a good correla-

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doi:10.1016/j.ejmech.2006.01.013

tion between the absorption in rats and human absorption was observed [5]. An in vivo experimental system is ideal for predicting the permeability of passively transported compounds with high accuracy. However, its use is hampered by the high cost of the large number of animals needed to obtain statistically significant absorption data. As a consequence, relatively large amount of sample are required (> 10 mg) for in vitro testing, which is typically not feasible in the early stage study of natural product drug discovery.

Alternatively, in vitro absorption models are commonly used to investigate transport mechanisms, classify permeability and predict in vivo absorption of drugs in humans for the reduced labor and experimental costs compared to in vivo animal studies [6]. Among the test systems, the everted rat intestinal sac (ERIS) model is a commonly used system to study drug absorption [7,8]. The non-everted sac model was originally used to evaluate drug transport mechanisms [9]. Later, Genty et al. [10] compared the permeability values of some actively transported molecules and passively absorbed compounds through both everted and non-everted sacs and found that the permeability was higher for actively transported molecules when the sacs were everted. The permeability of passive absorption drug diazepam remained the same whether the sacs were everted or not. These results suggested that the passive permeability of actively transported molecules can be determined through non-everted rat gut sacs [10]. Compared with everted sacs, information regarding the non-everted intestinal sacs model is limited. The primary goal of this study was to develop an in vitro, non-everted sac model to evaluate the intestinal permeability of the rat in order to establish the relationship between permeability of the rat intestine and human absorption and to predict the oral absorption, in humans of some selected natural compounds.

2. Materials and methods

Declaration: the animal experiments described herein were approved by the University Ethics Committee and the protocol complies with the recommendations of that committee.

2.1. Chemicals and reagents

The model drugs: caffeine, theophylline, ranitidine hydrochloride, chloramphenicol, and furosemide were provided by Shijiazhuang Pharmaceutical Group Co., Ltd.; aciclovir, norfloxacin were obtained from Zhejiang Cheng Yi Pharmaceutical Co., Ltd. (Wenzhou); cephalexin, acetaminophen, sodium diclofenac were available from Beijing Double-Crane Pharmaceutical Co., Ltd. (Beijing); and ketoprofen was a courtesy of the Southwest Synthetic Pharmaceutical General Factory (Chengdu).

Among the 13 natural compounds (Scheme 1), baicalin (1), hesperidin (2), naringin (3), rutin (4), genistein (5), genistin (6), puerarin (7), luteolin (8), and quercetin (9), were purchased from Shanxi Huike Botanical Development Co. Ltd. (Xi'an); matrine (11) was a gift from Guangdong Mingxing Pharmaceutical Co. Ltd. (Guangzhou). The other three compounds, namely, ampeloptin (10), fraxinellone (12), vitexin-7-glucoside (13) were isolated and purified in house. Their structures were fully confirmed by MS and NMR, with the purity by HPLC > 98%.

Hydroxypropyl-β-cyclodextrin (HP-β-CD) with a degree of substitution of 0.66 was purchased from Yiming File Chemical Co. Ltd.; acetonitrile and methanol was HPLC grade. Water used to prepare all solutions was doubly distilled. The Tyrode buffer contained 136.9 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 4.2 mM NaH_2PO_4, 1.2 mM $CaCl_2{\cdot}2H_2O$, 0.5 mM $MgCl_2{\cdot}6H_2O$, and 15 mM glucose.

2.2. Preparation of non-everted intestinal sacs

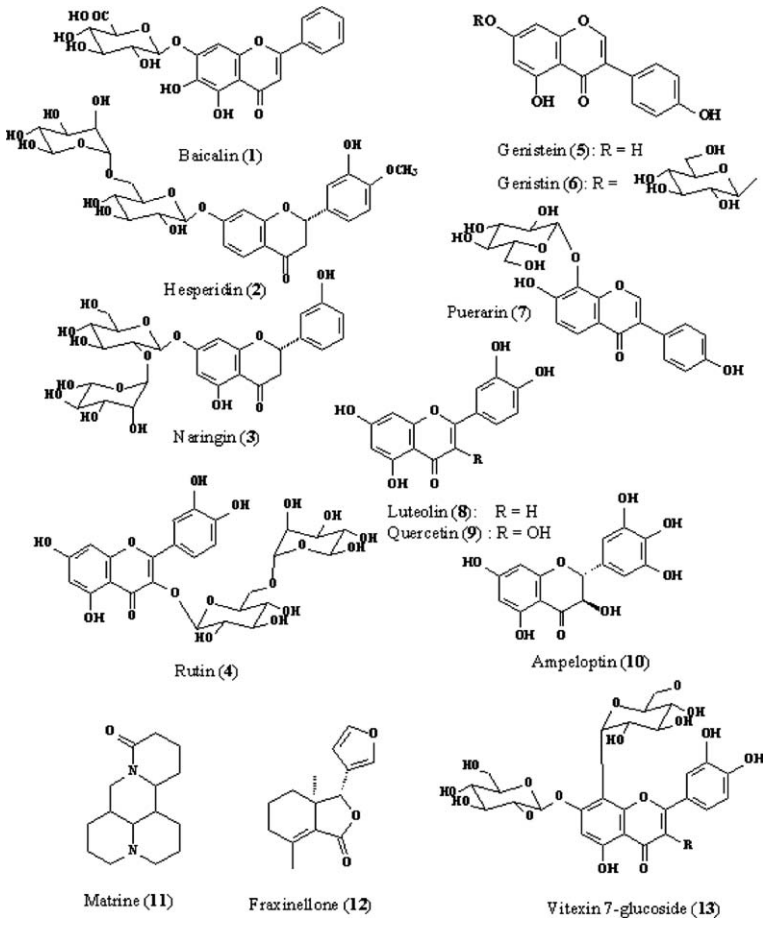
Male Sprague–Dawley rats, weighing approximately 280–320 g, were provided by the Central Animal Laboratory of China Pharmaceutical University. The animals were housed in a clean room with free access to food and water. Rats fasted overnight with free access to water were anesthetized by an intraperitoneal injection of urethane (1 g/kg). Upon verification of loss of the pain reflex, a midline longitudinal incision of 3–4 cm was made and the small intestine was located. The jejunum, approximately 35 cm distally from the ligament of Treitz, was excised and cannulated with a plastic tube. The inlet tube was connected to the reservoir containing fresh Tyrode buffer. The intestinal tract was washed in tyrode buffer at 37 °C by peristaltic pump feeding. The clean intestinal tract removed underlying mesenterium was prepared into approximately 5 cm long sacs with a volume of 1 ml by ligation. Each sac was cut and filled with oxygenated drug solution (1 ml, 500 μg/ml) via a blunt needle, and the other end tied.

2.3. Drug transfer in non-everted intestinal sacs

Each non-everted intestinal sac was placed in a glass bottle (50 ml) containing oxygenated tyrode buffer (10 ml). The sacs were maintained at 37 °C in a shaking water bath operating at 100 strokes per min and constantly gassed with O_2. Solution (10 ml) outside the sacs were taken every 20 min for 120 min and replaced with fresh oxygenated tyrode buffer (10 ml). The contents of the samples were subjected to HPLC analysis.

2.4. The selection of model drugs and the preparation of test solution

The drugs selected for the establishment of the relationship between rat intestinal permeability and human fraction absorbed possessed a range of physicochemical properties and molecular weights. In addition, their known human fraction absorbed data covered almost the full range and practical considerations leaned towards well-absorbed drugs because the value for high permeability was necessary for screening. Most of the drugs were known to be absorbed in humans through passive or partially passive transport mechanisms across GI membranes. Model drugs except furosemide and ketoprofen can be



Scheme 1. Structures of natural products tested for apparent permeability.

prepared into a solution at a concentration of 500 μg/ml by dissolving it in Tyrode buffer directly, furosemide and ketoprofen were done by adding a little dilute NaOH (0.1 M) solution. Hydroxypropyl-β-cyclodextrin (HP-β-CD) is considered a solubilizer possessing a high-solubilizing capacity and exhibiting minimal modulating impact on membrane integrity and absorption systems, such as passive diffusion and carrier-mediated permeation [11,12]. All of the poorly water-soluble natural compounds were solubilized with HP-β-CD in this study. Among the natural compounds, matrine and vitexin-7-glucoside can dissolve in Tyrode buffer at a concentration of 500 μg/ml, the others were solubilized by 20% (w/v) HPBCD, the solutions were filtrated and the initial concentration were determined. Sink conditions were essentially maintained during transport experiment, for all of the compounds tested, the highest concentration in the receptacle represented < 5% of the concentration inside the intestine.

2.5. Analytical methods

The concentrations of all of the drugs and compounds in the samples from the transport experiments were analyzed with a HPLC system equipped with a variable-wavelength ultraviolet detector (SPD-10A, Shimadzu, Kyoto, Japan), a 20 μl loop injection valve, and a reversed phase Versapack C_{18} (25 cm × 4.6 mm, 10 μm particle) column in conjunction with a pre-column insert. The following methods (Table 1) were applied to the measurement of the above-mentioned drugs and natural compounds.

2.6. Calculation of apparent permeability

The apparent permeability coefficient (P_{app}) of drugs and chemicals mentioned above was calculated from the following equation:

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A\ C_0} \quad (1)$$

Where dQ/dt is the steady-state appearance rate on the acceptor solution, A *is the* surface area of the intestinal sacs .C_0 is the initial concentration inside the sacs.

The sacs have a length of 5 cm and a volume of 1 ml; assuming they have a cylindrical shape, their inner diameter is 0.50 cm and the surface area is 7.85 cm^2 per sac. Although the method used takes no account of the microvilli and villi, this is of little consequence for the results of the present study, since the surface area of the intestine between the experiments was standardized, by performing an identical surgical procedure and always using 280–320 g male rats. Therefore, the

Table 1
HPLC methods for compounds studied

Drug/compound	Mobile phase (v/v)	Flow rate (ml/min)	Wavelength (nm)	Retention time (min)
Acyclovir	Methanol/water/acetic acid/triethylamine (20:80:0.02:0.002)	1.5	254	5.2
Norfloxacin	0.01 M potassium dihydrogen orthophosphate adjusted to pH 3.0 with phosphoric acid)/acetonitrile (87:13).	1.0	278	5.2
Ranitidine	0.77% Ammonium acetate/methanol (65:35)	0.8	320	6.0
Furosemide	0.02 M potassium dihydrogen phosphate and acetonitrile (80: 20) adjusted to pH 4.5 using phosphoric acid.	1.0	235	6.1
Acetaminophen	Methanol/water/acetic acid (35:65:1.5)	1.0	276	5.8
Chloramphenicol	Methanol/water (55:45)	1.0	246	4.7
Cephalexin	0.02 mol/l phosphoric solution (pH was adjusted to 3.0 with triethylamine): acetonitrile (80:20)	1.0	278	6.2
Sodium diclofenac	Methanol/water/acetic (80:20:0.5)	1.0	262	5.8
Ketoprofen	Acetonitrile/sodium dihydrogen phosphate (45:55)	1.2	284	6.5
Theophylline	Methanol/water (25:75)	1.0	258	6.4
Caffeine	Methanol/water/acetic acid (35:65:1.5)	0.8	272	3.9
Baicalin	Methanol/0.05% phosphoric acid (55:45)	1.0	272	6.6
Hesperidin	Methanol: 0.05% phosphoric acid (55:45)	1.0	280	7.2
Naringin	Acetonitrile/0.05% phosphoric acid (23:77)	1.0	283	5.6
Rutin	Methanol/0.05% phosphoric acid (50:50)	1.0	282	5.8
Genistin	Methanol/0.05% phosphoric acid (55:45)	1.0	254	7.2
Ampeloptin	Methanol/0.05% phosphoric acid (60:40)	1.0	258	6.2
Genistein	Methanol/0.05% phosphoric acid (60:40)	1.0	292	6.0
Matrine	Acetonitrile/0.05% phosphoric acid (6:94)	1.0	254	9.7
Puerarin	Methanol/0.05% phosphoric acid (30:70)	0.8	220	7.2
Luteolin	Methanol/0.05% phosphoric acid (60:40)	1.0	250	6.3
Quercetin	Methanol/0.05% phosphoric acid (60:40)	1.0	354	8.1
Fraxinellone	Methanol/water (75:25)	1.0	360	6.5
Vitexin-7-glucoside	Methanol/0.05% phosphoric acid (20:80)	1.0	220	8.2

permeability values obtained for the different compounds between assays are directly comparable.

2.7. Histological studies

Histological studies were performed to evaluate the effect of everted and non-everted intestinal sac models on the intestine. In the transport studies, everted and non-everted sacs filled with fresh tyrode buffer were operated under the same conditions. Before and after the experiment, some intestinal sacs were removed. The specimens were immediately fixed in 10% formaldehyde-saline solution and embedded in paraffin. Two to four micrometer sections were stained with hematoxylin and eosin (H and E) for histological evaluation.

3. Results and discussion

3.1. Relationship between rat intestinal permeability and human fraction absorbed

The apparent permeability of the entire selected model drugs, along with their literature data of their F_a values are presented in Table 2. The mean permeability values of the series of drugs examined range from 1.08×10^{-6} cm/s for acyclovir to 15.66×10^{-6} cm/s for caffeine. As expected, the permeability of acyclovir and norfloxacin, with lower bioavailability, are much lower than that of the drugs with high bioavailability, such as caffeine and ketoprofen. Cephalexin, which is absorbed by the H^+ -dependent dipeptide transport system, shows significantly lower permeability. Similar findings were reported by Yamashita et al. [13].

Fig. 1 illustrates the correlation between the human fraction absorbed and the permeability calculated with this non-everted intestinal sac model. As seen in Fig. 1, the rat intestinal permeability correlates to F_a closely through a trendline that consists of a steep slope region ($P_{app} < 5.6 \times 10^{-6}$ cm/s) and a plateau region ($P_{app} > 5.6 \times 10^{-6}$ cm/s) which was also characteristic in other models, including the Caco-2 cells versus F_a correlation

Table 2
Apparent permeability of non-everted intestinal sacs and human fraction absorbed for model drugs

Drugs	F_a (%)[a]	P_{app} ($\times 10^{-6}$ cm/s)
Acyclovir	21	1.08 ± 0.06
Norfloxacin	35	2.04 ± 0.45
Ranitidine	55	3.67 ± 0.18
Furosemide	60	4.65 ± 0.52
Acetaminophen	80	5.21 ± 0.14
Chloramphenicol	90	5.56 ± 0.59
Cephalexin	96	5.13 ± 0.45
Sodium diclofenac	100	8.80 ± 0.88
Ketoprofen	100	9.17 ± 1.93
Theophylline	100	12.17 ± 0.81
Caffeine	100	15.66 ± 1.06

[a] Literature F_a values are cited from Ref. [14].

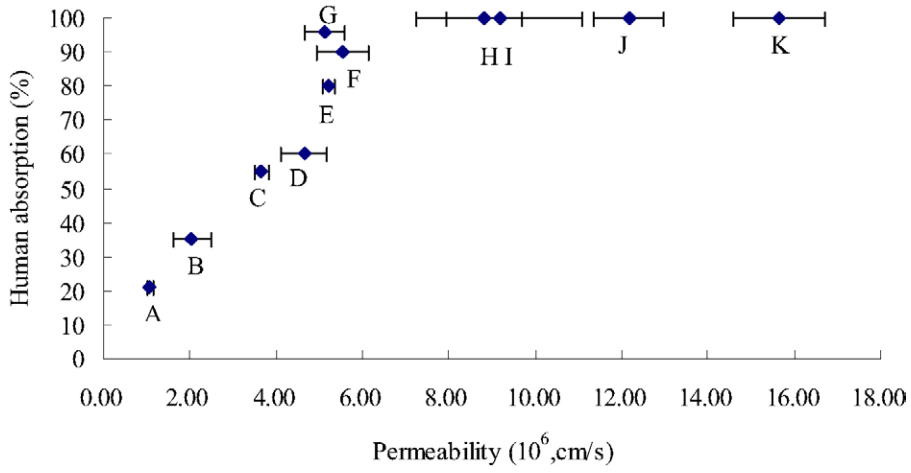


Fig. 1. Correlation between rat intestinal permeability and human fraction absorbed (mean value ± S.D., $N = 6$).
A: Aciclovir, B: Norfloxacin, C: Ranitidine, D: Furosemide, E: Acetaminophen, F: Chloramphenicol, G: Cephalexin, H: Diclofenac sodium, I: Ketoprofen, J: Theophylline, K: Caffeine.

[15], rat intestinal permeability versus F_a correlation [16–18] and the permeability obtained from regional perfusion studies in humans vs. F_a correlation [19]. In this process, the data for cephalexin was excluded because its permeability is lower in this experiment. These results suggest that, within certain limitations of drug solubility or stability in the GI tract, the model is suitable for predicting the human fraction absorbed of established drugs.

3.2. Estimating human fraction absorbed of natural compounds

Thirteen natural compounds with various biological activities were selected for assessing rat intestinal permeability. Although some of the compounds have been applied clinically, very limited pharmacokinetic information is available, particularly their intestinal uptake by humans and systematic bioavailability. There is a demonstrated need to clarify their absorption potential before they can be moved further in the drug discovery pipeline. Among these compounds, matrine (**11**) and vitexin-7-glucoside (**13**) have good water solubility whereas the others have poor water solubility. To evaluate the intestinal permeability of poorly water-soluble compounds, it is necessary to completely dissolve them in a medium and to avoid precipitation during experiments.

The apparent permeability of 12 test compounds, along with the estimated human fraction absorbed obtained by the above relationship is presented in Table 3. The permeability values of the 12 compounds cover a wide range, from 0.5×10^{-6} cm/s to 6.5×10^{-6} cm/s. According to the categories (High: Fraction absorbed > 66%; Medium: 33% < Fraction absorbed < 66%; Low: Fraction absorbed < 33%) [20], half of the test compounds had low permeability; four of the compounds [genistein (**5**), puerarin (**7**), luteolin (**8**), and matrine (**11**)] had medium permeability, only fraxinellone (**12**), and vitexin-7-glucoside (**13**) shown high permeability. The data indicate that most of the test compounds will have poor absorption in humans while **12** and **13** should have excellent absorption. The permeability of compounds with low or medium permeability should be enhanced in order to achieve therapeutic concentrations if they are intended for oral administration. Fraxinellone (**12**) therefore should be solubilized for oral administration due to its low solubility and high permeability. Vitexin-7-glucoside (**13**), on the other hand, with its high solubility and permeability, is worthy further investigation.

In this study, it was found that the permeability of quercetin (**9**) and some other flavonoids could not be determined by non-everted intestinal sac model as they are subject to metabolic conversion during their transport in the intestinal epithelial cells, consistent with several previous studies [21]. Quercetin was found to be metabolized rapidly and was below the detection limit in the sample within 30 min. Changing the tyrode solution to saline didn't change the situation. However, in another study to determine the permeability of quercetin (**9**) by caco-2 [22], it was shown that incubations for up to 60 min were acceptable and gave a linear transport of quercetin. The average recovery of quercetin was measured to be 67% and 84% in the experiments with apical and basolateral loading, respectively. These results indicate the notable differences of absorption and metabolism capacity between intestinal cells

Table 3
Apparent permeability and estimated human fraction absorbed of 13 natural compounds (mean value ± S.D., $N = 6$)

Test compounds	P_{app} ($\times 10^{-6}$ cm/s)	Estimated F_a (%)
Baicalin (**1**)	0.55 ± 0.06	< 21
Hesperidin (**2**)	1.00 ± 0.19	< 21
Naringin (**3**)	1.02 ± 0.01	<21%
Rutin (**4**)	1.38 ± 0.23	21–35
Genistin (**6**)	1.72 ± 0.27	21–35
Ampeloptin (**10**)	1.84 ± 0.37	21–35
Matrine (**11**)	1.88 ± 0.08	35–55
Genistein (**5**)	1.92 ± 0.36	35–55
Puerarin (**7**)	2.35 ± 0.27	35–55
Luteolin (**8**)	4.31 ± 0.49	50–60
Quercetin (**9**)	Data not available	
Fraxinellone (**12**)	4.71 ± 0.06	60–80
Vitexin-7-glucoside (**13**)	9.50 ± 0.19	> 100

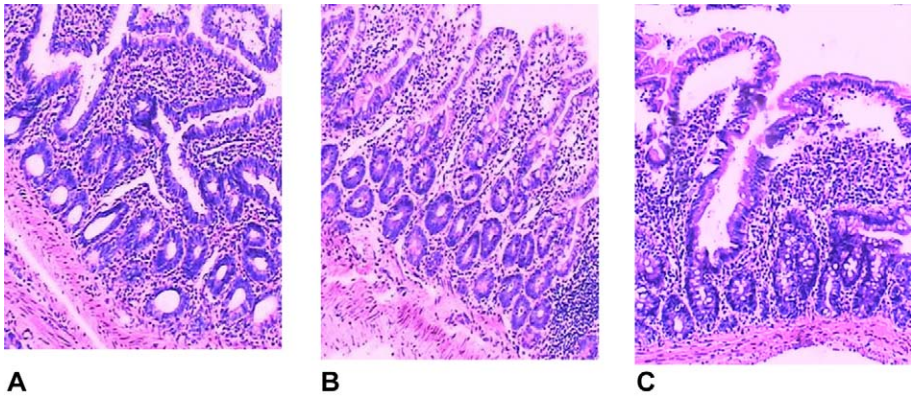


Fig. 2. Histological section of rat intestine: A, control; B, everted intestinal sac; C, non-everted intestinal sac (H and E stain, 100-fold magnification).

and caco-2 cells, and highlight the need for different absorption models for drug screening and profiling.

3.3. Histological studies

The light microscopic pictures of the intestine are presented in Fig. 2. A typical cross section of the intestine before the experiment is depicted in Fig. 2A, when the tissue sample was directly kept in formalin. Fig. 2B and C show the figures of the intestine at the end of the transport experiment by the everted and non-everted models. It is apparent that the villi at the mucosal surfaces and in the intestinal lumen were relatively intact, and no significant presence of inflammatory cells was observed in these samples. No disruption of the epithelium was observed. Histological studies showed no evidence of significant changes in the structure of the intestine when the everted and non-everted models were compared to controls.

4. Conclusion

The non-everted intestinal sac model is a simple and rapid technique for the study of intestinal drug transport. It is also possible to perform studies with other animal intestines, thus providing a methodology to compare the permeability values across species. It is capable of predicting both the extent and the rate of drug absorption, providing information with regard to preabsorptive metabolism or degradation, thereby possibly predicting the human oral absorption of drugs from in vitro measured data. Comparing with everted sac model, the non-everted intestinal sac model has some advantages: the preparation of non-everted sacs was simple; the amounts of drug required for the study are relatively small; samples can be collected successively and frequently; the collected samples are analytically clean which facilitates quantitative analysis; morphological damage caused by everting the intestinal tissue can be avoided; gut sacs can be made when the rats are alive, and consequently the time employed for the transport experiment may be longer than that of everted gut sacs. Taken together, this study demonstrates that non-everted intestinal sacs may be used as an alternative method to everted gut sacs for the evaluation of in vitro permeability in rats and estimating human absorption.

Acknowledgments

This work was supported by a grant from the State Ministry of Education (n° 104200), a grant from the Jiangsu Province Government, China, awarded to Bo-yang Yu; and a grant from the National Natural Science Foundation of China (n° 30472197).

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