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ORIGINAL ARTICLE

Evaluation of gastric toxicity of indomethacin acid, salt form and complexed forms with hydroxypropyl-β-cyclodextrin on Wistar rats: histopathologic analysis

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ABSTRACT

Indomethacin (IM) is a non-steroidal anti-inflammatory drug which inhibits prostaglandin biosynthesis. It is practically insoluble in water and has the capacity to induce gastric injury. Hydroxypropyl-β-cyclodextrin (HP-β-CD) is an alkylated derivative of β-CD with the capacity to form inclusion complexes with suitable molecules. IM is considered to form partial inclusion complexes with HP-β-CD by enclosure of the *p*-chlorobenzoic part of the molecule in the cyclodextrin channel, reducing the adverse effects. The aim of this paper is to evaluate the gastric damage induced by the IM inclusion complex prepared by freeze-drying and spray-drying. A total of 135 Wistar rats weighing 224.4 ± 62.5 g were put into 10 groups. They were allowed free access to water but were maintained fasted for 18 h before the first administration until the end of the experiment. IM acid-form, IM trihydrated-sodium-salt and IM-HP-β-CD spray and freeze-dried, at normal and toxic doses, were administered through gastric cannula once/day for 3 days. Seventy-two hours after the first administration, the animals were sacrificed and the stomachs collected and prepared for morphological study by using the haematoxylin-eosin technique. Lesion indexes (rated 0/4) were developed and the type of injury was scored according to the severity of damage and the incidence of microscopic evidence of harm. Microscopic assessment demonstrated levels of injury with index one on 10–25%. The type of complexation method had different incidence but the same degree. The results show that IM inclusion complexation protects against gastric injury, reducing the incidence and the maximum degree of severity from 4 to 1, with a better performance of the spray-dried complex.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs), which mechanism of action involves the inhibition of the prostaglandin biosynthesis, have the capacity to induce gastric injury, it being one of its most common adverse effects [1]. There are two essential characteristics that determine the NSAIDs ulcerogenicity: the kinetics of the gastrointestinal absorption of the drugs and the fact that they can make a multiple attack to some or to all of the mucosa's defence mechanisms. The ulcerogenic degree achieved depends on those defence mechanisms, on the biochemical processes affected and on the power of the effect of the drugs in these processes. Indeed for a drug to

show an ulcerogenic effect, it must be present in the mucosa's cells in concentration and for time enough to break the toxicity mechanisms. Generally, when NSAIDs are administered orally, a large concentration in the gastric mucosa's cells is attained; nevertheless, when the administration is parenteral, some NSAIDs that suffer enterohepatic recirculation may also produce lesions of the mucosa or ulcers [2–7].

Indomethacin (IM) is a NSAID used for the treatment of rheumatoid arthritis, ankylosing spondilytis, osteoarthritis and for the closure of patent ductus arteriosus (PDA) on newborns. From a physicochemical standpoint, IM, which is slightly acidic, is poorly water soluble, and soluble only at pH 12, a pH at which significant hydrolysis of the molecule occurs [8,9]. IM can be administered by injection, tablet, capsule or suppository. When administered by any route, IM can produce gastrointestinal side effects, including irritancy, and can cause ulceration of the stomach and intestine, with this effect more severe following oral administration [3].

After oral administration of IM to rats, in doses that vary between 2.9 and 20 mg/kg, the plasmatic peak is attained at 3 h. IM suffers hepatic metabolization and shows linear elimination kinetics, being excreted through the urine in the unaltered form and under the form of metabolites conjugated with glucuronic acid [10–12]. The pharmacokinetic behaviour of IM enables three possible ways by which it can enter in contact with the mucosa in the gastrointestinal tract [7,13]: a *topic phase* – after ingestion, at the gastric level, and during absorption in the small intestine and in the large intestine; a *systemic way* – which occurs as far as the drug enters the vascular compartment and distributes itself along the organism, being able to reach the apical cells in the gastric mucosa; an *excretion through the biliar way* – exposing the small intestine and the stomach to a contact phase, by reflux of the duodenal content [7].

Cyclodextrins (CDs) are cyclic, non-reducing, water-soluble oligosaccharides. Their unique properties are attributable to their apolar cavities. The products of CDs hydrolysis include glucose and maltooligosaccharides, which are known to be readily fermentable by colon anaerobes to yield fatty acids and flatus gases, among other products. They have the capacity to fill their cavity with the molecule of another substance, forming 'inclusion complexes'. Inclusion complexes of poor water soluble molecules with alkylated CDs present a better water solubility, a greater rate of dissolution and a more efficient absorption after oral administration than uncomplexed molecules [14,15].

Hydroxypropyl-β-cyclodextrin (HP-β-CD) is an alkylated derivative of β-CD. The hydroxyalkylation generates amorphous mixtures, with a water solubility usually greater than 50% w/v, and forming stable complexes with many drugs [16]. Performed clinical studies have demonstrated that HP-β-CD is considered to be non-toxic if the daily dose is inferior to 16 g, and have also shown that it has no mutagenic potential, being either embryotoxic or teratogenic [17].

IM is considered to form partial inclusion complexes with HP-β-CD by enclosure of either the *p*-chlorobenzoic part or the ring of the indol unit of the molecule in the CD channel [18–20]. These inclusion complexes have the capacity to enhance IM's solubility and stability in aqueous solution and to reduce the gastrointestinal adverse effects [18,21].

PDA is the persistence of a normal foetal structure between the left pulmonary artery and the descending aorta. Persistence of this foetal structure beyond 10 days of life is considered abnormal. IM is a drug of choice for closure of the ductus administered by intravenous route [22,23]. The overall idea of our work is to investigate the possibility of using an inclusion complex form of IM to treat PDA by oral administration, with the aim of overcoming, on one hand, the problems of administering intravenous medications to newborns, and on the other hand, the impossibility of using the oral route due to the hydrolysis of IM that occurs at gastric basic pH of newborns of 6–8, and also due to gastrointestinal toxicity.

Therefore, the purpose of the present study is to evaluate in Wistar rats the gastric damage induced by the IM inclusion complex prepared by freeze-dried (FD) and spray-dried (SD) methods, compared with IM sodium salt form and IM acid form.

MATERIALS AND METHODS

Drug products

IMaf was kindly provided by Merck Sharp & Dohme (Lisboa, Portugal); IMss tri-hydrated was purchased under the name of Indocid® (Coimbra, Portugal); HP-β-CD, molecular weight of 1300 and medium molar substitution degree of 0.39, was obtained from Jassen Biotech (Beerse, Belgium); IM complexes with HP-β-CD were prepared by FD and SD methods.

Doses selection

Two different doses of the testing products were administered: a therapeutic dose (TD), defined as the dose equivalent to the one used to treat newborns with PDA –

0.2 mg/kg – and a toxic dose (TxD), defined as the dose referred in the literature as having the capacity to induce lesions without causing mortality – 10 mg/kg [7,24]. The calculation of the dose equivalent to the TD was based on the application of the conversion factor recommended by the Food and Drug Administration when the animal in study is the rat (accessible in: http://www.fda.gov/cder/cancer/animalframe.htm). The IM dose present in the complexes obtained by each of the complexing methods was calculated from the percentage of IM included in the HP-β-CD, based on the results of the technological study performed – the freeze-dried complex (CFD) contained 17.3% of IM and the spray-dried complex (CSD) contained 24.3% of IM [25]. HP-β-CD dose, used as control, was the same amount of HP-β-CD contained on TxD sample, corresponding therefore to the higher percentage. It was assumed that the dose of IMss to be administered for the TxD would be the double the quantity of the TD, because this dose is already considered as having a toxic capacity.

Product solutions were prepared as follows: TxD (IMaf and IMss) = 2 mg/mL (30 mg IM/15 mL water); TD (IMaf and IMss) = 46.28 μg/mL (1 mg IM/21.6 mL water); TxD (CSD) = 8.24 mg/mL (123.6 mg CSD/15 mL water); TD (CSD) = 190.46 μg/mL (4 mg CSD/21 mL water); TxD (CFD) = 11.56 mg/mL (173.4 mg CFD/15 mL water); TD (CFD) = 267.5 μg/mL (5.35 mg CFD/20 mL water); TxD (HP-β-CD) = 9.56 mg/mL (95.6 mg CD/10 mL water); Water = 5 μL/g animal.

Animals

This study was carried out on 2-month old male and female Wistar rats weighing 224.4 ± 62.5 g (Harlan Iberica, Barcelona, Spain). The rats were housed in a local *bioterium* under standard laboratory conditions, which include a temperature of 20–24 °C, relative humidity about 50–60% and a controlled 12-h light cycle beginning early in the morning. Animals were allowed free access to water but the food was withdrawn from them 18 h before the beginning of the first administration. Animal experimentation in this study was conducted in accordance with the European guidelines for the care and use of laboratory animals (86/609/EEC) and the project was approved by the Portuguese Veterinary General Division.

Experimental design

Gastric toxicity studies in animals

Existing studies with IM in rats show that the location of the lesions on the rat depends on the feeding or fasting conditions experienced [26–28]. In continuous feeding rats, IM induces the development of ulcers mainly in the antrum and in the duodenum. Therefore, it becomes a good model for the study of the induction of adverse effects by NSAIDs in the superior gastrointestinal tract, through inhibition of the prostaglandin biosynthesis in the rat's stomach [29,30]. The small intestine, on the other hand, is especially sensitive to the long fasting [7,31]. Subsequently, it can be inferred that the fasting itself will not affect the gastrointestinal organ in study – the stomach. Accordingly, by using rats kept in fasting for a long time, it can be assured that eventual lesions appearing in the stomach of the rats are not due to the fasting or feeding conditions experienced but are certainly induced by IM, which evaluation is the goal of the present study.

Experimental procedures

A total of 135 animals were randomly distributed into 10 groups. Ten rats per group were used for the TxD of IMaf, complexed IM forms and negative control group (water) and 20 rats per group were used for the TD of IMaf, complexed IM forms and for the CD group used as control. The number of animals used for the IMss form was a minor sample due to limitation of the available product: eight rats for the TD of IMss group and seven rats for the TxD of IMss group.

The product solutions were orally administered by resorting to a cannula [32], in a volume of 0.005 mL/g of body weight, every 24 h over three consecutive days, by analogy with the frequency of administration of the IM for the pharmacological treatment of the PDA [24]. The food was withdrawn from the animals 18 h before the beginning of the first administration, having been allowed free access to water during the entire experiment [8,24,26]. Twenty-four hours after the last administration the animals were sacrificed. Stomach and small intestine were macroscopically observed for lesion evidence and the stomachs were collected for histopathological study procedure.

Weight evaluation

Each animal was weighed prior to food withdrawn and before being sacrificed. For each rat, the calculation, in percentage, of the weight variation in view of the total weight of the animal, before and after the treatment, was performed, in accordance with the following equation [33]: loss of weight (%) = $((W_i - W_f)/W_i) \times 100$, where W_i represents the initial weight (rat's weight 18 h before the beginning of the experience) and W_f means final

weight (rat's weight 72 h after the administration of the first dose, at the time of its sacrifice).

The hypotheses that can be used to explain the weight variation in function of the performed intervention are that the weight can vary depending on the type of product administered, on the administered dose or on the lesion's index caused by the administered products. This last hypothesis will be evaluated in the stomach's histopathologic study. The variable used to test these hypotheses was the percent variation of the weight.

Histopathologic study of gastric mucosa fragments

Sample preparation

For the histopathological study, each stomach previously collected was opened over its great curvature and gently washed with isotonic sodium chloride. Mainly in the antrum and duodenum, macroscopic evaluation showed areas with lesions. Transversal fragments of these areas on the stomach were fixed in phosphate-buffered formaldehyde (pH 7.2), embedded in paraffin, and routine 5-μm sections were prepared. Five serial sections of each block were routinely stained with haematoxylin and eosin and evaluated for severity of damage by light microscopy and photographed [34–36].

Gastric index lesion evaluation

The calculation of the gastric index lesion in the morphological study was made according to a lesion level of severity scale developed by us, with the attribution of the following degrees: 0 – normal morphological aspect; 1 – vascular and/or oedema congestion; 2 – superficial ulceration or single small ulcer; 3 – more than one ulcer or just a very large ulcer or small areas of necrosis or of haemorrhage; 4 – large necrosis or great haemorrhage. The study of the morphological lesion was performed in each animal and, when more than one type of lesion was observed in the same animal, the more serious lesion was considered for index classification.

Statistical analysis

The SPSS 11.0 (Statistical Package for the Social Sciences) for Windows was used in the present data processing.

Weight evaluation

Parametric methods were used whenever the samples revealed adherence to normality, through the analysis of histograms and the result of the Kolmogorov–Smirnov test, as well as through the variation factor and the asymmetry data. The ANOVA analysis was used when the comparison of more than two independent variables from an interval scale was performed and, whenever a significant difference was verified, the lower significant difference was determined between the various pairs of groups. The Student's *t*-test was used to compare the average of two independent variables. Both methods of average comparison considered the difference as statistically significant for a value of $P < 0.05$. The Wilcoxon test was used for several coupled variables from an interval scale and non-normal distribution. A statistical significance was considered when $P < 0.01$.

Lesion assessment

For the lesion's index study, the Kruskal–Wallis test was used. Every time that a significant difference was observed for an H value, it was followed by the application of the Mann–Whitney U-test. The statistically significant difference was considered for $P < 0.05$.

The evaluation of the incidence's statistical meaning and the lesion's severity was made between the intervention groups and the control one (water) or the comparative one (TD of IMaf), either for TD or TxD.

Weight comparisons

In order to evaluate if there is a variation relationship between two coupled variables from an interval or ordinal scale and without adherence to normality, the Spearman correlation method was used, showing a statistical significant difference when $P < 0.01$.

RESULTS

Weight evaluation

The weight evaluation performed during the study revealed a reduction of weight in all groups. Comparisons and correlations between the initial and the final weight, in the totality of the animals and by groups of drug products, were performed in order to study the behaviour of the animals' weight before and after the treatment. The Wilcoxon test showed that there is a significant diminishing of initial weight in all the animals. The same test was applied to evaluate in which groups of products a more significant weight variation was verified, having been concluded that it is significant in all the groups. The result of the Spearman correlation test presented a value of $r = 0.976$ statistically significant, which means that there is a strong positive linear association, with the final weight directly proportional to the initial weight (simultaneous variation) (*Figure 1*).

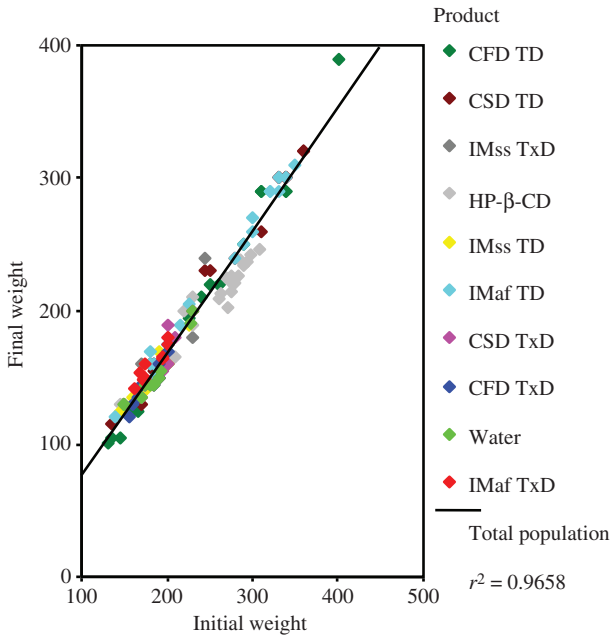

Figure 1 Scatter diagram for the correlation between initial and final weight.

Lesion assessment

Morphologic changes

All animals presented macroscopic evidence of lesion in the small intestine. The microscopic observation of the stomach's sections revealed the presence of the following types of morphologic changes: ulcer (*Figure 2*), haemorrhage, vascular congestion, necrosis or necrosis with inflammatory infiltrates (*Figure 3*), oedema and exulceration (*Figure 4*). *Table I* presents a synthesis of the results of the microscopic observation of the studied tissue fragments. The results are expressed in number of

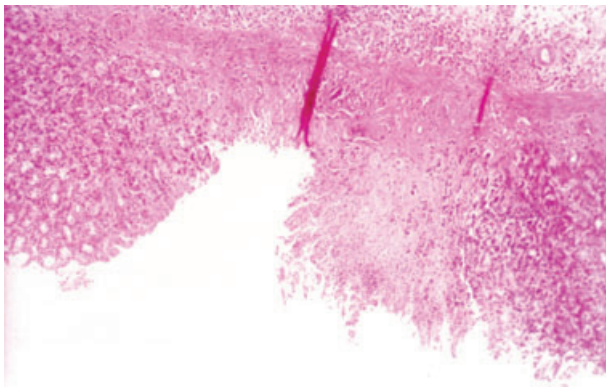

Figure 2 Histological section of gastric mucosa. Exulceration in one animal submitted to the administration of IMss in the therapeutic dose (H&E, original magnification ×50).

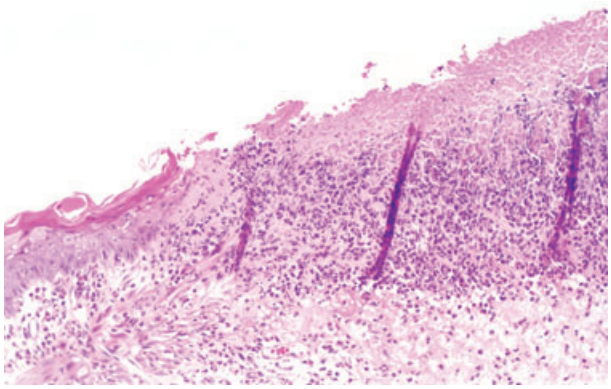

Figure 3 Histological section of gastric mucosa. Extensive area of necrosis and inflammatory infiltration in one animal submitted to the administration of IMaf in the toxic dose (H&E, original magnification ×50).

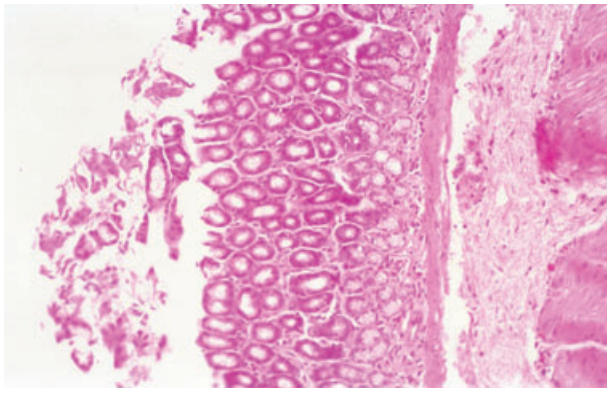

Figure 4 Histological section of gastric mucosa. Gastric ulcer in one animal submitted to the administration of IMaf in the therapeutic dose (H&E, original magnification ×50).

Table I Microscopic morphologic changes.

Product	Dose	Presence (PR = Am/At)	LLS	GIL (PR = AIL M/At)
IMaf	TD	17/20 (0.85)	4	2/20 (0.10)
	TxD	10/10 (1.00)	3	6/10 (0.60)
IMss	TD	4/8 (0.50)	4	1/8 (0.125)
	TxD	4/7 (0.57)	4	1/7 (0.142)
CFD	TD	5/20 (0.25)	1	5/20 (0.25)
	TxD	2/10 (0.20)	1	2/10 (0.20)
CSD	TD	2/20 (0.10)	1	2/20 (0.10)
	TxD	1/10 (0.10)	1	1/10 (0.10)
Water	0.005 mL/kg	0/10 (0.00)	0	0
HP-β-CD	TxD	0/20 (0.00)	0	0

GIL, gastric index lesion; PR = Am/At, ratio between animals with presence of morphologic change and total number of animals of each group; PR = AIL M/At, ratio between animals with maximal index lesion change and total number of animals of each group; LS, level of lesion severity.

animals in which the presence of morphologic changes was detected, as a proportion of the total number of animals of each group and the relative proportion.

Non-complexed indomethacin

The IMaf groups of therapeutic and TxDs presented morphologic lesions in the stomach's wall. In the TD group, exulcerations and some ulcers were mainly observed, sometimes deep, like the ones in *Figures 2* and *4*; in the TxD group, in addition to the lesions observed in the therapeutic group, the presence of large haemorrhages, necrosis of the wall and inflammatory infiltrates (*Figure 3*), with predominance of mononucleated cells, were frequently observed.

The group of IMss showed the presence of morphologic changes equivalent to those of the IMaf group, both for the therapeutic and TxDs (*Figure 2–4*).

Complexed indomethacin form

There was no evidence of morphologic lesions of levels 2, 3 or 4 in any group administered with complexed IM, with whichever dose, as exemplified in *Figure 5*. Only level 1 lesion was found in few animals.

Water and hydroxypropyl-β-cyclodextrin

Both for water, as general negative control, and for HP-β-CD, as negative control of IM complexed forms, there is no evidence of morphologic lesions, as exemplified in *Figure 5*.

Gastric index lesions

The proportion of animals that evidence any type of morphologic lesion is shown in *Table II*. The results showed a significant reduction of the incidence of lesions, as well as its severity. The type of lesion classified as level 1, observed in the animals administered with IM complexed forms, is considered to have no clinical significance and to be reversible.

We have also compared the proportion of animals with adverse events in all intervention groups, with IM TD and TDx, as control groups (*Table III*), to evaluate the incidence reduction attained with the complexation methods and with the sodium salt form. The major lesion reduction of animal proportion was observed for CSD both on TD (8.5) and TDx (10.0), followed by the CFD group for the TD (3.4) and the TDx (5.0). The reduction observed for IMss group were less significant (1.7) and are equivalent for both doses.

Comparative analysis of lesion's index between products

The Kruskal–Wallis test originated a value of $H = 28.642$ for TD and $H = 24.525$ for TxD, both presenting

Table II Proportion of animals that showed gastric morphologic modifications.

Product	Dose	Evidence of morphologic modifications	Proportion of animals with adverse events
IMaf	TD	17/20	0.85
	TxD	10/10	1.00
IMss	TD	4/8	0.50
	TxD	4/7	0.57
CFD	TD	5/20	0.25
	TxD	2/10	0.20
CSD	TD	2/20	0.10
	TxD	1/10	0.10
Water	0.005 mL/kg	0/10	0.00
HP-β-CD	TxD	0/20	0.00

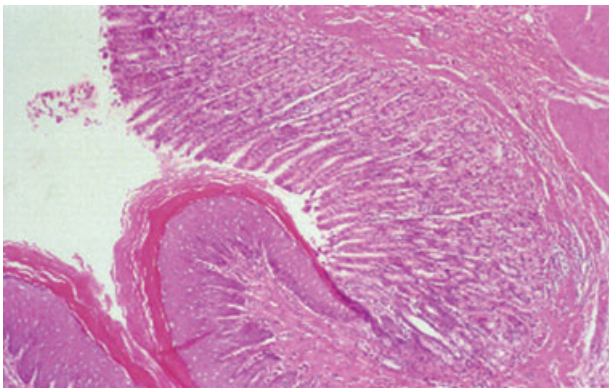

Figure 5 Histological section of gastric mucosa. No evidence of morphologic changes in one animal submitted to the administration of CFD in the therapeutic dose (H&E, original magnification ×50).

Table III Evaluation of lesion reduction achieved with the complexation methods and with the sodium salt form, by comparison with indomethacin therapeutic dose and toxic dose, as control groups.

Dose	CG	PAAE	IG	PAAE	CG/IG
Therapeutic	IMaf	0.85	IMss	0.50	1.7
		0.85	CFD	0.25	3.4
		0.85	CSD	0.10	8.5
Toxic	IMaf	1.00	IMss	0.57	1.7
		1.00	CFD	0.20	5.0
		1.00	CSD	0.10	10.0

PAAE = Proportion of animals with adverse events, from Table II; CG = control group; IG = intervention group.

a significant difference. This statistical test was followed by the application of the Mann–Whitney *U*-test, whose *P* values showed significant differences between the complexed forms and the IMaf, either in the TD or in the TxD.

Weight comparisons

Comparison of the percent of weight variation by groups of products

The ANOVA analysis showed a significant difference between the various groups used. The post hoc lower significant difference test did not allow establishing a causality relation in terms of the products under study, since all of the comparisons presented significant variations of percent loss of weight, in relation to initial weight.

Comparison of the percent of weight variation by used dose

For this analysis, the animals were regrouped considering the administered dose. The Student's *t*-test applied to the analysis of the percent of weight variation averages revealed that the difference between the two groups considered – TD and TxD – is not significant.

Percent of weight variation and lesion's index

With the intent of testing if the percent variation of weight can be related to the observed lesion's index, rats were regrouped according to the lesion's index determined and in function of the performed comparison and correlation non-parametric tests. The Spearman test revealed the existence of a weak negative association between the lesion's index and the weight percent variation ($r = -0.282$; $P = 0.001$). The data dispersion analysis showed that a greater part of the animals are distributed in the area of the lesion's index zero, presenting a percent weight variation that spreads out from the minimum to the maximum value observed, and that in the indexes that represent higher lesion severity, the weight percent variation oscillates between average values (*Figure 6*). The comparison of the percent weight variation between the animal groups with different lesion's indexes was performed through the Kruskal–Wallis test. Since this type of analysis does not allow using samples with a number inferior to seven, the animals of the lesion's index level three and four were merged. The application of the test to the totality of animals revealed the existence of a significant difference between groups. To determine between which pairs of groups the difference was significant, multiple combinations and Mann–Whitney *U*-test were performed. Significant differences were verified between the animals with index lesions zero and one and between animals presenting index lesions zero and three.

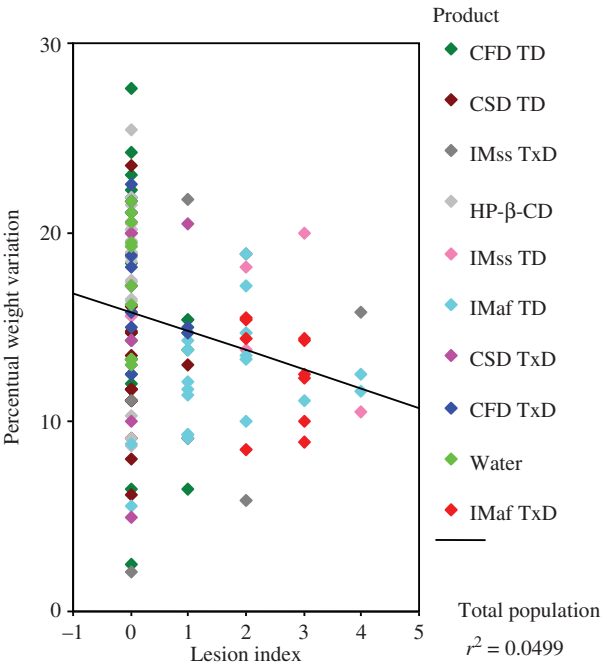

Figure 6 Scatter diagram for the correlation between percentual weight variation and morphologic lesion index.

DISCUSSION

Taking into consideration that the lesion's index obtained for the IM complexed forms was the lowest (one = vascular congestion and/or oedema) and believed to be transitory and clinically non-significant, this study corroborates that complexes, when administered on three consecutive days, present a beneficial effect over either IMaf or IMss, both with TD or TDx, either in terms of incidence or in terms of lesion's severity. Additionally, IMaf and IMss do not present a significant difference for the TD or for the TDx, which is what might have been expected for IMss, the tablet's form, since the sodium salt form should give some protection to the mucosa from the direct contact with the acidic form of IM. It is also relevant to point out that there was no evidence of gastric lesions with HP-β-CD or with water. However, macroscopic lesions were observed at the level of the small intestine like in the experiments published in the literature [7,31], confirming that the fasting condition leads to this kind of effect, since all animals presented them.

The relationship between the weight variation and the lesion's index described in the bibliography is

controversial. Some authors present results that show a diminishing of weight with an increase of toxicity while not observing a decrease in weight in the animals to which the CD complex was administered [33]. Others obtained a weight increase in the animals treated with CD complexes [8]; other authors impute to the CDs an effect on the reduction of weight gain, fat deposition and triglycerides blood level [37]. The above considerations have been also attributed to the fact that the HP-β-CD is not hydrolysed by the amylases, and therefore, not originating the metabolites (malto-dextrines, maltose and glucose) that could be absorbed and could promote the increasing of weight by its nutritional value [38].

From the analysis of the linear regression line obtained (*Figure 6*), we can consider that, in view of the variables, about 5% ($r^2 \times 100$) of the weight percent variation is explained through the lesion's index, with the remainder explained by other factors external to this study, and that it was not possible to corroborate any of the data of the studies of the other previously mentioned authors, since all animals showed weight reduction.

CONCLUSION

Histopathologic study of gastric mucosa fragments of CSD through spray-dried method showed a significant reduction in the proportion of animals with gastric lesion regarding IMaf, being the reduction on the group of CFD less significant. However, these lesions were considered not clinically significant and transitory.

From these results we can conclude that CSD obtained by spray-dried method presents a better safety profile. This behaviour can be attributed to its dissolution profile [39], thus sustaining the hypothesis that the dissolution parameters are more adequate to the objective of gastric toxicity reduction, since it presents less percentage of dissolved IM at 5 and 30 min (33.6 ± 1.2 and 93.2 ± 2.6, respectively) then CFD (92.6 ± 1.8 and 98.9 ± 1.2, respectively), if taking into account that lesion can be caused by IM high local concentrations.

Under these circumstances, dissolution parameters modification and enhancing absorption by complexation can result in a reduction of drug capacity to induce gastric lesion [2].

In this way, the non-immediate release of IM from spray-dried complex, can indicate that the main quantity of IM was released in the small intestine, being therefore effective for gastric protection, confirming what some authors [16,40–45] have published, that CDs do not suffer modification at gastric level and that the quantity of released drug results from a kinetic balance between the various entities present in solution.

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