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Effects of *Solanum glaucophyllum* toxicity on cell proliferation and apoptosis in the small and large intestine of rabbits

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**Abstract**

Vitamin D regulates mineral homeostases and enterocyte proliferation and differentiation. Hypervitaminosis D generates changes in cell proliferation, differentiation and apoptosis in several organs. We analysed morphometric parameters and proliferative and apoptotic indices in the intestinal epithelium of rabbits with hypervitaminosis D induced by the chronic treatment with the calcinogenic plant *Solanum glaucophyllum*. Rabbits were treated for 15 or 30 days. A group was treated for 15 days and led to possible recovery for 30 days. Another group was nutritionally restricted for 30 days. Morphological, morphometric, proliferative and apoptotic changes were found in the treated animals. Mild atrophy and reduced proliferation was found in the jejunum and ileum. Apoptosis increased in the crypts of the ileum and in the superficial epithelium and crypts of the rectum. Most of the alterations were partially recovered. The possible involvement in these changes of the hypervitaminosis D-like state induced by *S. glaucophyllum* is discussed.

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1. Introduction

Vitamin D has been associated with increased absorption of dietary calcium and phosphate (Wesley et al., 2006; Bikle, 2007) and enterocyte proliferation and differentiation (Suda et al., 1990; Newmark and Lipkin, 1992; Menard et al., 1995; Holt et al., 2002) in the gastroenteric tract. It induces the expression of genes involved in environmental intestinal toxin detoxification (Kutuzova and Deluca, 2007), and in the preservation of the mucosal barrier integrity (Kong et al., 2008).

Cell proliferation, differentiation and apoptosis continuously occur in the intestine, and they are tightly regulated to ensure epithelial renewal (Potten, 1991; Potten et al., 1997). These processes can be altered under starvation (Holt et al., 1986; Chappell et al., 2003), fasting (Park et al., 2008) and chronic psychological stress in rats (Boudry et al., 2007), as well as under *Escherichia coli* infection in rabbits (Wada et al., 1997; Heczko et al., 2001).

*Solanum glaucophyllum* (*S. glaucophyllum*) (synonym of *Solanum malacoxylon*) is a calcinogenic plant responsible for enzootic calcinosis in ruminants of South America, a cause of considerable economic losses in Argentina, Brazil and Uruguay (Worker and Carrillo, 1967). The toxic principles, mainly contained in the leaves, consist of vitamin D analogues, mostly 1,25(OH)2D3, associated with glycoside derivatives. The chronic ingestion of this plant induces a hypervitaminosis D-like state (Wasserman, 1974; Dallorso et al., 2001; Mello, 2003). Spontaneous intoxication occurs in ruminants when the active principle is released into the rumen. The intoxication of non-ruminant species, such as pigs (Campero and Odriozola, 1990) and horses (Ruager and Gimeno, 1977), shows that the toxic principle does not need to suffer structural modifications such as the hydrolysis of the glycosides accounted in the rumen, as it has been previously suggested (Boland et al., 1986; Mello and Habermann, 1992). Intoxicated animals show mineralization in heart, arteries, lungs and kidneys, join erosions, stiffness, painful gait, kyphosis, anorexia and loss of body condition. In severe cases, emaciation is also observed (Worker and Carrillo, 1967), although the cause remains unknown. Rabbits are useful as experimental model. They are highly susceptible to experimental treatment with *S. glaucophyllum* in a way similar to the natural intoxication that occurs in ruminants (Dallorso et al., 2001; Gimeno et al., 2004; Fontana and Zanuzzi, 2007; Zanuzzi et al., 2008; 2010).

Experimental studies performed in animal models treated with *S. glaucophyllum* have shown changes in cell proliferation, differentiation and apoptosis in aorta, lungs (Barros and Gimeno, 2000; Portiansky et al., 2002), skin (Gimeno et al., 2000; 2004), thymus, lymph nodes and spleen (Fontana and Zanuzzi, 2007; Fontana,
2.2. Treatment with S. glaucophyllum and experimental groups

*S. glaucophyllum* leaves were collected in the locality of Chascomús (Buenos Aires, Argentina), an area of high incidence of enzootic calcinosis, during springtime. The active principle is stable for up to four years (Puche et al., 1981). The leaves were air-dried, powdered with a blender and administered as pellets, which consisted of a mix of standard diet, tap water and 125 mg/kg of powdered leaves, a dose chosen based on previous works (Dallorso et al., 2001; Comús, 1990). All morphological measurements were conducted by two blinded researchers.

2.3. Histological studies

All rabbits were necropsied and samples of small and large intestine were collected. Samples were obtained from jejunum (middle region, 1 m from the pyloric sphincter), ileum (10 cm from the ileocecal junction) and rectum, and then rinsed in 0.2 M PBS, fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin wax. Paraffin blocks were sectioned on a semiautomatic microtome (LEICA RM2245, GmbH, Nussloch, Germany) and six 3-μm-thick non-contiguous (100 μm apart) sections of each intestinal region of every tested animal were routinely stained with haematoxylin and eosin (HE) according to Bancroft and Stevens (1990). All morphological measurements were conducted by two blinded researchers.

2.4. Morphometric studies

Sections stained with HE were examined under a light microscope. A minimum of 10 well-oriented crypt villous units or crypts of each section from jejunum, ileum and rectum were analysed for each animal. The length and width of villi and crypts were measured. Images of each sample section were captured from a microscope (Olympus BX50, Tokyo, Japan), with objectives of 10× or 40× magnification, using an attached digital video camera (Olympus DP71, Tokyo, Japan) connected to a computer containing an image analysis program (ImagePro Plus, v6.3, Media Cybernetics, USA), and stored in RGB TIFF 24 bits format.

2.5. Proliferation study

Sections (3 μm thick) were mounted on slides coated with g-methacryloxypropyltrimethoxy-silane (M6514, Sigma, St. Louis, MO, USA), passed through a decreasing graded alcohol scale and incubated with 0.03% H2O2 in methanol (purum 99.0%) for 30 min at room temperature. Sections were then rinsed twice in PBS and exposed to microwave antigen retrieval using a buffer citrate solution (pH 6.0) (Taylor et al., 1996). Sections were then incubated with 1% BSA in PBS for 30 min, followed by overnight incubation with anti-Ki67 antibody (monoclonal mouse anti-human Ki-67 antigen Clone MIB-1, ready-to-use, DakoCytomation, Carpinteria, CA, USA). The EnVision® detection system + HRP system labelled anti-mouse polymer (DakoCytomation) was applied for 30 min. Sections were then rinsed three times in PBS for 5 min each time. Liquid 3,3-diaminobenzidine tetrahydrochloride (DAB) (DakoCytomation) was used as chromogen and Hill’s haematoxylin for counterstaining. Control negative sections were prepared by omitting primary antibody. A section of feline squamous cell carcinoma was used as a positive control. At least 700 cells per section were counted using a 40× objective and the number of labelled and non-labelled epithelial cells was recorded. Proliferation index was expressed as the percentage of labelled cells over the total epithelial cells using the following formula:

\[
\text{Proliferation index} = \frac{\text{number of epithelial labelled cells}}{\text{total number of epithelial cells}} \times 100
\]

2.6. Apoptosis study

In the HE-stained sections apoptotic cells were identified using the following morphological criteria: cell shrinkage, acidophilic cytoplasm and condensation and fragmentation of nuclear chromatin. Apoptosis was confirmed by the use of the modified terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling technique (TUNEL kit ApopTag Chemicon International, Temecula, CA, USA). We followed...
the manufacturer's suggested protocol. All TUNEL labelled samples were counterstained with methylene green. TUNEL protocol included negative controls excluding the TdT step of the original method. As positive control we used the positive sample provided by the commercial kit.

To calculate the apoptotic index in the villi we measured the perimeter of the villi and counted the number of apoptotic cells along that perimeter. The apoptotic index was expressed as:

\[
\text{Apoptotic index} = \frac{\text{number of apoptotic epithelial cells in the villi}}{100 \times \text{total number of epithelial cells}}
\]

To calculate the apoptotic index in the crypts in the three anatomic segments and in the superficial epithelium of the rectum we counted at least 700 cells per slide using a 40× objective. The apoptotic index was expressed as:

\[
\text{Apoptotic index} = \frac{\text{number of apoptotic epithelial cells in the crypt}}{100 \times \text{total number of epithelial cells}}
\]

2.7. Statistical analysis

An analysis of variance (ANOVA) with one factor was used. Significance was assumed at values of \(p < 0.05\). The Fisher least significant difference (LSD) test was used for the post hoc comparisons. For these comparisons, the \(p < 0.01\) level was used to define significance between groups.

3. Results

3.1. Clinical signs and macroscopic lesions

All the treated animals from groups A and B reduced the ingestion of food after 24–48 h. Compared to control animals, all the groups showed a significant reduction in their body weight index from day 7 up to the end of the experiment (Fig. 1). Treated animals also showed other clinical signs, such as diarrhoea, rhinitis, conjunctivitis or external otitis. Animals from group C showed clinical signs only during the treatment time, and partially recovered body weight during the remaining 30 days. Animals from group D also showed a gradual body weight reduction. In contrast to treated animals, they were more aggressive and hyperkinetic.

At necropsy, animals from groups A and B showed reduction of adipose tissue compared to control animals, muscular atrophy and

Fig. 1. Body weight variations in S. glaucophyllum-treated animals. Note the progressive reduction of body weight index with time in treated animals. All the groups showed a significant reduction in their body weight index in comparison to control group (\(p < 0.05\)).

Fig. 2. Morphological changes of the intestine. A–C: jejunum; D–F: ileum; G–I: rectum. A, D and G: control group. B, E: group B. H: group A. C, F and I: group C. Samples of jejunum and ileum of treated animals show atrophy of the villi. A mild recovery is observed in the same intestinal tracts in group C. Multifocal slight atrophy was observed in the rectum. All sections were stained with H–E. Bar = 1 mm.
calcification of the cardiovascular system (tricuspid and mitral valves, inner surface of aorta and pulmonary arteries), lungs and renal cortices. Animals from group C only showed calcification of the cardiovascular system.

3.2. Histological studies

The mucosa of the jejunum, ileum and rectum from the treated groups during 15 (group A) and 30 days (group B) showed atrophy (Fig. 2). In jejunum and ileum the degree of atrophy of the villi was about 30%. In some animals lymphangiectasia extended up to the tunica muscular (Fig. 3). The mucosal layer of the animals from group D appeared slightly atrophic with oedema and lymphangiectasia in the lamina propria.

3.3. Morphometric studies

3.3.1. Jejunum

Villous length was significantly reduced in groups A as compared to control and D groups, whereas villous width was significantly higher in group C in comparison to control group. Crypt width increased in group C as compared to all groups except group D (Fig. 4).

3.3.2. Ileum

Villous length (Fig. 4) was significantly reduced in group A as compared to control and D groups. Villous width increased in group C compared to group A. Crypt length was significantly reduced in group A compared to control animals.
3.3.3. Rectum

No significant differences were found in crypt length or width neither between treated animals nor with controls (Fig. 4).

3.4. Proliferation and apoptosis studies

3.4.1. Jejunum

Proliferation index was significantly decreased in animals from group B compared to controls and group D (Fig. 5). No significant difference in the apoptotic index was observed between groups (Fig. 6).

3.4.2. Ileum

Proliferation index was significantly reduced in group A compared to the rest of the groups (Fig. 5). There was a significant increase in the apoptotic index of the crypts in group A compared to the rest of the groups (Fig. 6).

3.4.3. Rectum

The proliferation index was significantly reduced in group D compared to group B (Fig. 5). The apoptotic index of the superficial epithelium and crypts was significantly increased in group A in comparison to group C and D (Fig. 6).

4. Discussion and conclusions

Vitamin D regulates cell proliferation, differentiation and apoptosis in the intestine (Menard et al., 1995; Díaz et al., 2000; Biol-N’Garagba et al., 2002; Holt et al., 2002; 2006). Several studies have shown that 1,25 (OH)₂ vitamin D₃ inhibits cell proliferation in normal rectal mucosa (Thomas et al., 1992), adenomatous polyps (Holt et al., 2006) and colonic and rectal cancer cell lines (Díaz et al., 2000). These findings have supported its use in the prevention and treatment of hyperproliferative disorders (Bikle, 2007; 2008).

Changes in cell proliferation, differentiation and apoptosis have been reported in several organs under a hypervitaminosis D state (Barros and Gimeno, 2000; Gimeno et al., 2000; Portiansky et al.,

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**Fig. 5.** Ki-67 proliferation index in the intestine. The proliferation index was reduced in some S. glaucophylum-treated animals. In the jejunum, significant differences (*) were observed between group B vs. control and D groups. In the ileum, significant differences (#) were observed between group A and the rest of the groups. In the rectum significant differences (&) were observed between group D vs. group B. The figure shows the means of the experimental groups and the 99% confidence interval on the treatment means. *, #, & = p < 0.01.

**Fig. 6.** Apoptosis of epithelial cells. In ileum crypts (*) significant differences vs. the remaining groups were observed. In the superficial epithelial cells and crypts of the rectum (*) significant differences vs. groups C and D were found. The figure shows the means of the experimental groups and the 99% confidence interval on the treatment means. * = p < 0.01.
was reduced after 30 and 15 days of treatment, respectively. In were affected. Cell proliferation in the jejunum and in the ileum
were reported in goats, sheep (Liesegang et al., 2002; Gimeno et al., 2003) and in the mucosa and lamina propria of young rats fed with low
protein content diet (Rodrigues et al., 1985). According to Drozdowski and Thomson (2006) dynamic morphological parameters,
such as enterocyte proliferation and migration, are associated with intestinal adaptation to internal and external environmental
stimuli. Modifications in the proliferation and apoptosis may explain the changes in the size of villi and crypts found in several pathological conditions.

In the present study, we observed morphological and morpho-
metrical alterations in the intestines of rabbits fed with the calcin-
ogenic plant \textit{S. glaucophyllum}. Besides, cell proliferation and death
were affected. Cell proliferation in the jejunum and in the ileum
was reduced after 30 and 15 days of treatment, respectively. In
contrast, in the rectum the changes in apoptosis seemed to play
a primary role. In other experimental studies, variations in prolifera-
tion rate within intestinal regions have also been reported, e.g. in
rats subjected to thermal stress (Greant et al., 1988) and in the
colon of mice fed with a vitamin D-deficient diet (Sadava et al., 1996).
Specific adaptive responses of each anatomical region and differ-
ences in the expression of vitamin D receptors within the gastroin-
testinal tract have been reported in goats, sheep (Liesegang et al.,
2007; Riner et al., 2008) and rabbits (Duncan et al., 1984). Vari-
ations in the number and distribution of vitamin D receptors in
the intestine might also explain the results obtained in the present
study. In addition, the hormonal and metabolic profile of animals
from group C during and after the treatment period, and the differ-
ences in the mechanism of adaptation or recovery of each intesti-
nal section could account for their partial recovery.

The response of the intestinal mucosa to different injuries or
stressors reveals the great plasticity of this organ to adapt to most
of them, a property known as enteroplasticity (Drozdowski and
Thomson, 2006). In some circumstances the response to similar
injuries might differ. Holt et al. (1986) observed a hyperplastic re-
sponse in the ileum of nutritionally restricted animals, and a re-
duced cell proliferation in the duodenum. Other authors (Chappell et al.,
2003) found a decreased cell proliferation in the proximal intestine of rats subjected to progressive nutritional
restriction, with changes in cell death only during complete fast-
ing. In contrast, the apoptosis was increased in the crypts of ani-
mal under intermittent water avoidance stress (Boudry et al.,
2007).

The results from group D differed from those of control or trea-
ted groups according to the intestinal section analysed and the
measured parameters. Thus, the alterations found in the treated
animals might result from the reduction in food ingestion, from a
specific response to \textit{S. glaucophyllum} treatment or a combination of
both. Taking into account the present results and the well-
known vitamin D antiproliferative effects in the intestine (Thomas et al.,
1992; Holt et al., 2006; Bikle, 2007; 2008), it is possible that
the changes observed in affected animals were primarily induced
by \textit{S. glaucophyllum} treatment.

Atrophy and changes in cell proliferation and death have also
been reported in knockout mice for \textit{FGF-23} and \textit{Klotho} genes that
exhibit a hypervitaminosis-D like status (Razzaque and Lanske,
2006; Lanske and Razzaque, 2007). They show hyperphosphatemia,
hypercalcaemia, atherosclerosis, emphysema, kyphosis, alopeci-
a, loss of body weight, osteopenia/osteoporosis, hypogonadism,
soft tissue calcifications and atrophy of several organs, such as thymus
and spleen (Razzaque and Lanske, 2006; Medici et al., 2008).
Most of the aforementioned clinical signs are frequently found in
naturally and experimentally \textit{S. glaucophyllum}-intoxicated animals.

Particularly, in the intestine of \textit{FGF-23} and \textit{Klotho}-knockout mice,
intestinal mucosa and villi are atrophic (Razzaque and Lanske,
2006; Lanske and Razzaque, 2007). The reduced proliferation and
the increased apoptosis explain those morphological alterations
(Razzaque and Lanske, 2006; Lanske and Razzaque, 2007; Medici
et al., 2008). The changes described in these knockout mice have been
attributed to the hypervitaminosis D status, and considered
premature ageing-like features (Razzaque and Lanske, 2006; Lanske
and Razzaque, 2007; Medici et al., 2008). We also found signifi-
cant differences in the proliferation index of the small intestine
and in the apoptotic index of the ileum and rectum in the treated
animals. In line with these observations, the similarity between the
results of our model and those from transgenic mice further support
the involvement of vitamin D in the changes found in the treated
rabbits.

The reduced body weight in the treated animals supports that
reported in naturally intoxicated cows by Worker and Carrillo
(1967), and in experimentally intoxicated rabbits by Dallorso et al.
(2001). It has been shown that high calcium levels reduce
weight gain (Davies et al., 2000; Heaney et al., 2002). Thus, the
intestinal atrophy, the reported modifications on the glycosylation
process (Zanuzzi et al., 2010), and the hypercalcemic state found in
the treated rabbits (Fontana, 2010) could lead to alterations in the
digestive and absorption processes, and explain the gradual and
progressive loss of weight.

Vitamin D has immunomodulatory effects (Bikle, 2008).
Although we did not evaluate the specific impact of hypervitamin-
osis D state on the adaptive and innate immunity the clinical signs
and lesions found in the treated animals strongly suggest local or
systemic immunomological impairment. In addition, atrophy of lym-
phoid organs, changes in proliferation and apoptosis, and hyper-
plasia and hypertrophy of Paneth cells, were other changes
reported in cattle and rabbits treated with \textit{S. glaucophyllum} (Fonta-
nata and Zanuzzi, 2007; Zanuzzi et al., 2008; Fontana et al.,
2009; Fontana, 2010). The otitis, rhinitis, conjunctivitis or diar-
rhoea described in this study have been previously reported in rab-
bits treated with \textit{S. glaucophyllum} by several authors (Dallorso
et al., 2001; Gomar, 2006; Fontana, 2010) who did not observe ana-
atomopathological characteristic lesions suggestive of diseases in-
duced by infectious agents, such as rotavirus, coronavirus or
Escherichia coli.

In conclusion, the findings described in this work show that
\textit{S. glaucophyllum} treatment induced a hypervitaminosis D-like state
that alters cellular proliferation and apoptosis in the intestinal epi-
thelium. These results will help to better understand the clinical
signs observed in naturally intoxicated animals.

5. Conflict of interest statement

All the authors declare that there is no actual or potential con-
lict of interest including any financial, personal or other rela-
tionships with other people or organizations within three years of
beginning the submitted work that could inappropriately influence,
or be perceived to influence, their work.

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