Nephrotoxicity of ibandronate and zoledronate in Wistar rats with normal renal function and after unilateral nephrectomy

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\textbf{A R T I C L E  I N F O}

Article history:
Received 18 January 2015
Received in revised form 28 April 2015
Accepted 30 April 2015
Available online 11 May 2015

Keywords:
Bisphosphonates
Renal toxicity
Ibandronate
Zoledronate
Unilateral nephrectomy

\textbf{A B S T R A C T}

A previous animal study compared the nephrotoxic effect of ibandronate (IBN) and zoledronate (ZOL), but interpretation of these study results was limited because of the model of minimal nephrotoxic dosage with a dosage ratio of 1:3. The present study investigated the nephrotoxicity of ibandronate and zoledronate in a 1.5:1 dose ratio, as used in clinical practice and compared the nephrotoxicity in rats with normal and with mildly to moderately impaired renal function. We compared rats with normal renal function (SHAM) and with impaired renal function after unilateral nephrectomy (UNX), treated either with ibandronate 1.5 mg/kg, zoledronate 1 mg/kg or placebo once (1×) or nine (9×) times. Renal function and markers of tubular toxicity were measured over a 27 week period. After last bisphosphonate treatment the rats were sacrificed and kidneys examined histologically. All bisphosphonate treated animals showed a significant tubular toxicity, which was temporary except in the ZOL-UNX-9×-group. Also the renal function was only transiently reduced except in the ZOL-UNX-9×-group. Histologically, bisphosphonate treatment led to cortical tubulopelithelial degeneration/necrosis and medullary tubulopelithelial swelling which were slightly more pronounced in ibandronate treated animals, when compared to zoledronate treated animals, especially with impaired renal function. In contrast to the previous study we found a similar nephrotoxicity of ibandronate and zoledronate in rats with normal renal function. In rats with impaired renal function the peak of toxicity had not even been fully reached until end of experiment in the zoledronate treated animals. The peak of toxicity seems to be more severe and delayed in rats with impaired renal function compared with rats with normal renal function.

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1. Introduction

Bisphosphonates are potent osteoclast inhibitors used in bone diseases with an increased osteoclast activity like osteoporosis, Paget’s disease or tumor induced bone disease. Bisphosphonates have been reported to have only few side effects, although in the literature there is some indication of acute renal failure and even kidney failure requiring dialysis after repeated bisphosphonate infusions [1–4]. An other adverse effect is osteonecrosis of the jaw [5,6]. The present paper focuses on renal toxicity.

After entering circulation, bisphosphonates are rapidly taken up in bone or excreted unchanged via the kidney. Bone takes up 40–60% of the dose [7–9]. The remaining 60–40% are eliminated within 24 h almost exclusively via the kidney. Bisphosphonates are not metabolized [10]. The exact route of renal elimination has still not been clearly determined. In rats during renal excretion about 0.5% of the administered drug remains in the kidney tissue with the risk of accumulation. This might be the reason for renal toxicity after repeated dosage. In rats with mild renal insufficiency the renal tissue level increases significantly after repetitive treatment.

\textbf{Abbreviations}: 1×, group with only one injection; 9×, group with nine injections; \textalpha\textsuperscript{-}GST, \textalpha\textsuperscript{-}glutathione S-transferase; \textbeta\textsuperscript{-}NAG, \textbeta\textsuperscript{-}N-acetyl-glucosaminidase; AUC, area under the curve; BW, body weight; CREA, creatinine; GFR, glomerular filtration rate; HE, hematoxylin and eosine; IBN, ibandronate; NoOP, not operated; PBS, phosphate buffered saline; PL, placebo; SHAM, sham operated; SPF, specified pathogen free breeding; UNX, unilaterally nephrectomized; ZOL, zoledronate.

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\textit{http://dx.doi.org/10.1016/j.phrs.2015.04.016}
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compared to rats with normal renal function, but it is unknown whether this finding is associated with an increased renal toxicity [11].

Ibandronate and zoledronate are the most potent bisphosphonates for treatment of cancer related bone disease and tumor induced hypercalcemia. Due to an increased incidence of cancer in older people, bisphosphonate therapy is used in many cases in patients with impaired kidney function due to secondary disease (e.g., diabetes, hypertension), age related renal insufficiency or cancer induced renal disease [12,13].

Additionally, some chemotherapeutic agents (e.g., cisplatin, gemcitabine) are nephrotoxic [14]. Further drugs should therefore not deteriorate renal function. In rats with mildly impaired renal function the renal tissue level of ibandronate increases significantly after repetitive treatment in comparison to rats with normal renal function [11]. The influence of a mildly to moderately impaired renal function on the nephrotoxicity of zoledronate was not investigated so far, but zoledronate is contraindicated in severe renal insufficiency (stage IV-V, GFR < 30 ml/min). There are also no data about renal tissue concentrations of zoledronate.

To date, there are no reports about renal toxicity of ibandronate in patients, but it is assumed, that the different bisphosphonates have a different nephrotoxic potential [15–17].

To the best of our knowledge there are no clinical studies, comparing the nephrotoxic side effects of the different bisphosphonates in cancer patients. One animal experiment compared the nephrotoxic effect of zoledronate and ibandronate and demonstrated an increased incidence of renal failure and tubular damage in zoledronate treated rats [18]. But interpretation of these study results was limited because of the model of minimal nephrotoxic dosage, in that for both drugs a dosage was used which induced a minimum nephrotoxic damage after a single infusion. The dosage was 1 mg/kg BW for ibandronate and 3 mg/kg BW for zoledronate (dosage ratio 1:3, whereas in clinical use a dosage ratio of 1.5:1 is used). Moreover, the study was only performed in rats with normal renal function. Therefore, the present study investigated the nephrotoxicity of ibandronate and zoledronate in a 1.5:1 dosage ratio, as used in clinical practice, and compared the nephrotoxicity in rats with normal and first time also with mildly to moderately impaired renal function.

2. Methods

96 female Wistar rats (HsdHan:Wist) from a specified pathogen free breeding (SPF) (Harlan GmbH, Roßdorf, Germany), aged 8 weeks and weighing 210±10 g at arrival, were randomly assigned to thirteen groups of eight or four animals, respectively. The rats were assigned to three treatment groups (ibandronate (IBN), zoledronate (ZOL) and sodium chloride 0.9% (placebo, PL)). Each treatment group was then subdivided into one group with a single injection (1×) and one group with nine repeated injections (9×). Finally every subgroup was additionally divided into sham operated rats (SHAM) with normal renal function and unilaterally nephrectomized rats (UNX) with impaired renal function. In the placebo treated rats a non-operated group (NoOP) was carried along as a baseline for comparison of possible effects related to the experimental procedures. Fig. 1 gives an overview over all groups and time points of measurements.

The rats were group-housed (4 animals/cage) in macrolon cages (1800 cm²) on soft bedding (Lignocel 3/4 S, Ssniff Spezialdiäten GmbH, Soest, Germany) provided with nesting material. The had ad libitum access to water and pelleted standard rodent diet; 19% protein, 3.3% fat, 4.9% fiber, energy 12.8 MJ/kg, (Ssniff Spezialdiäten GmbH, Soest, Germany).

After an adaptation period of two weeks animals underwent unilateral nephrectomy (UNX) or sham surgery (SHAM) as described in a recent publication [11]. From the age of 16 weeks the 9×-groups received a total of nine injections of ibandronate 1.5 mg/kg BW, zoledronate 1 mg/kg BW or sodium chloride 0.9% 1 ml, not BW adapted (placebo), at a dosing interval of 3 weeks. All injections were administered into the tail vein over a time period of maximum 30 s. The 1×-groups received only one injection of ibandronate, zoledronate or placebo at the age of 38 weeks. All animals were sacrificed, perfused and necropsied 3 days after the last injection.

The dosage was calculated on the base of the previously published paper from Pfister et al., who demonstrated a minimal nephrotoxic dosage of zoledronate with 1(-3) mg/kg BW [18]. The ibandronate dosage was adjusted in a 1:1.5 ratio (zole- dronate/ibandronate), as used in clinical practice.

Fig. 1. Overview of treatment groups and animal numbers per group and time points of measurement of creatinine clearance, α-GST and β-NAG at weeks 15, 22, 31 and 40.
Serum samples were taken before first injection (week 15 of age), as well as 3 days after the third injection (week 22), sixth (week 31) and last injection (week 40). Urine samples were collected in metabolism cages at the same date over a 16 h period. In rats with only one injection serum samples and urine samples were taken before the injection and 3 days after the injection. Creatinine was measured in blood serum and urine with an enzymatic color test (CREA Plus #11775669, Roche Diagnostics, Germany) with a Hitachi® 911 autoanalyzer (Roche Diagnostics, Germany). Creatinine clearance was adjusted to 100 g body weight. Further parameters measured in the urine samples were α-glutathione S-transferase (α-GST) and β-N-acetyl-glucosaminidase (β-NAG), as markers for renal tubular damage. α-GST was determined with an enzyme immuno assay (#BIO64RAT, Biotrin International, Germany), as β-NAG with an enzymatic color test (#875406, Roche Diagnostics Germany) with an Advia®1650 autoanalyzer (Bayer HealthCare AG, Germany). All results were compared with the corresponding placebo-group (e.g. IBN-SHAM-9× and ZOL-SHAM-9× were compared with PL-SHAM-9×) and also with the baseline value of the same group for significant differences. All statistical analysis were calculated with SAS (Version 9.1; SAS Institute, Cary, NC). For the statistical evaluation we used ANOVA with a Bonferroni correction. P-values <0.05 were rated as significant.

For unilateral nephrectomy and for sacrifice deep anesthesia was achieved by intraperitoneal injection of ketamine 100 mg/kg BW (Hostaket®, Hoechst Roussel Vet) and xylazine 5 mg/kg BW (Rompun® 2%, Bayer AG). For systemic perfusion and sacrifice, once deep anesthesia achieved, the abdominal aorta was punctured in the distal third, the vena cava opened, and animals were then perfused with cold phosphate buffered saline (PBS) for approximately three minutes. Kidneys were then removed and weighed.

For histological examination the kidney samples were fixed in 2% paraformaldehyde for 24 h, dehydrated and embedded in paraffin. Transversal sections (approximately 3 µm) were stained with hematoxylin and eosin and were evaluated for the full spectrum of all possible histopathological findings by light microscopy, by a veterinary specialist for toxicologic pathology. The severity of lesions was graduated on a semiquantitative scale as follows:

<table>
<thead>
<tr>
<th>Grade</th>
<th>Severity</th>
<th>Number</th>
<th>Size</th>
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<tbody>
<tr>
<td>1</td>
<td>Minimal</td>
<td>Very few</td>
<td>Very small</td>
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<tr>
<td>2</td>
<td>Mild</td>
<td>Few</td>
<td>Small</td>
</tr>
<tr>
<td>3</td>
<td>Moderate</td>
<td>Moderate number</td>
<td>Moderate size</td>
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<tr>
<td>4</td>
<td>Marked</td>
<td>Many</td>
<td>Large</td>
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</table>

Group mean severity grades for each finding were calculated by adding the observed scores within a group and dividing by the number of animals present in that group.

In the statistical analysis every 9× treated group was compared with the corresponding 1× treated group and the corresponding placebo treated group.

The study was previously approved by the animal protection board of the medical faculty Mannheim, university of Heidelberg.

3. Results

One rat of the PL-SHAM-9× group had to be sacrificed prematurely because of a severe tail necrosis. All other rats survived until study termination and were evaluated.

3.1. Serum and urine biochemistry

There were no significant differences in creatinine clearance, α-GST and β-NAG between the PL-SHAM-9×-group and the PL-NoOP-9×-group. The creatinine clearance was significant lower in all UNX-groups compared with the SHAM-groups, confirming mild renal impairment. In the placebo groups the creatinine clearance increased over the time. There was no significant difference in the creatinine clearance between the IBN-SHAM-9×-group and the PL-SHAM-9× group over the time. The IBN-UNX-9×-group developed a significant decrease of the creatinine clearance at week 31 (p = 0.007 vs. PL-UNX-9×-group), but this difference resolved at week 40. The final creatinine clearance reached the baseline level approximately. In the ZOL-SHAM-9×-group the creatinine-clearance decreased significantly at week 22 (p = 0.038 vs. PL-SHAM-9×-group) and week 31 (p = 0.014 vs. PL-SHAM-9×-group), but recovered also at week 40 to the baseline level approximately. The ZOL-UNX-9×-group showed a significant lower creatinine clearance compared to the PL-UNX-9×-group at week 31 (p = 0.0069) and 40 (p = 0.0024), because this group had no increase of the creatinine clearance over the time. Like the PL-UNX-9×-group. For the results of all creatinine clearances see Table 1.

β-NAG did not change significantly in any group, at any time point. α-GST increased significantly in the IBN- and ZOL-SHAM-9×-group at weeks 22, 31 and 40. This increase was significantly higher in the IBN-SHAM-9×-group (p = 0.007 week 22 and p = 0.045 week 31 vs. ZOL-SHAM-9×-group) (Table 2). However, in contrast to the IBN-SHAM-9×-group α-GST remained significantly elevated in the ZOL-SHAM-9×-group compared to the baseline levels. Also in the IBN/ZOL-UNX-9×-groups α-GST remained significantly elevated compared to the baseline levels, but with the tendency for decline in the IBN-UNX-9×-group. On the other hand, in the ZOL-UNX-9×-group the α-GST was increasing constantly over all measurements. α-GST increased also significantly in both groups with a single

<table>
<thead>
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<th>Table 1</th>
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<tr>
<td>Creatinine clearances of the different groups over the time. Every group was compared with the baseline value of the same group (e.g. IBN-UNX-9× week 40 with IBN-UNX-9× week 15), with the corresponding placebo group (e.g. IBN-UNX-9× week 40 with PL-UNX-9× week 40) and with the corresponding treatment group (e.g. IBN-UNX-9× week 40 with ZOL-UNX-9× week 40). UNX and SHAM groups were not compared.</td>
</tr>
<tr>
<td>Treatment</td>
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<tr>
<td>Treatment</td>
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<td>Time</td>
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Significant vs. baseline: *p < 0.05.
Significant vs. corresponding PL-group p < 0.05; *p < 0.01.
Significant vs. corresponding treatment (ZOL/IBN) group: #p < 0.05, #p < 0.01.
4. Discussion

Like in a previous study, we could demonstrate some renal toxicity after injections of a minimal nephrotoxic dose of ibandronate and zoledronate, which was more pronounced in animals with mild to moderate renal insufficiency (UNX-groups), compared to rats with normal renal function [18, 19]. In the placebo groups an age related increase of creatinine clearance was seen, which may be attributable to an increased transglomerular passage of creatinine and a gain of muscle mass in older rats [20]. In contrast to the SHAM-groups, the UNX × -groups treated with zoledronate or ibandronate did not show this increase of creatinine clearance over the time. After a short decrease (week 31) of creatinine clearance which was significant in the IBN-UNX × group and not significant in the IBN-SHAM × and ZOL-SHAM × groups, the renal function reached a level similar to baseline at the end of the study. However, there were no significant differences in any group compared to the comparable placebo groups, demonstrating only a transient nephrotoxicity in the IBN-UNX/SHAM × and the ZOL-SHAM × group, severe enough to impair the renal function. The decrease of renal function after UNX was about 25–30%, which was described in previous studies with an UNX rat model [21, 22].

It is known that healthy humans, having donated one kidney for transplantation, show a decrease of 25–30% of baseline renal function [23]. The limitation of this model is the fact, that otherwise healthy people, as well as rats can compensate the loss of one kidney partly, which is not the case in patients with an age-related renal insufficiency.

During the course of the study α-GST, which is produced in the proximal renal tubules and serves as a marker for acute tubular damage, increased with a similar course in UNX- and SHAM-groups [24, 25]. However, after unilateral nephrectomy the baseline α-GST level was lower, when compared to sham-operated animals, because of the lower number of tubular cells in these animals.

The ibandronate treated animals showed the highest increase of α-GST at weeks 22 and 31, but with a decrease at week 40. Also the ZOL-SHAM-9 × -group had a similar course of α-GST but significantly lower than the IBN-9 × -groups. In all bisphosphonate treated groups, except the IBN-SHAM-9 × -group, the α-GST remained significantly elevated at week 40, demonstrating a persisting tubular damage. In contrast to the other bisphosphonate treated animals, the ZOL-UNX-9 × showed a continuous significant increase over time, compared to baseline, with no tendency of decrease over the 27 week study period.

This finding demonstrates a more acute tubular toxicity after ibandronate treatment but with a recovery over the time despite...
Fig. 2. Renal histology, HE staining. (a) Inner cortical region of an animal from group PL-NoOP-9× showing normal appearance of tubules and glomeruli. (b) Inner cortical region of an animal from group ZOL-UNX-9× showing tubuloepithelial degeneration/necrosis with epithelial vacuolation, disordered epithelium, detached epithelial cells and karyopyknosis. (c) Medullary region of an animal from group PL-NoOP-9× showing regular collecting ducts. (d) Medullary region of an animal from group IBN-UNX-9× showing enlarged epithelial cells in several collecting ducts.

Table 3
Results of histopathological evaluation. The results are separated according their treatment (column) and the findings (line).

<table>
<thead>
<tr>
<th></th>
<th>Ibandronate</th>
<th>Zoledronate</th>
<th>Placebo</th>
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<tbody>
<tr>
<td></td>
<td>SHAM-9× UNX-9× SHAM-1× UNX-1× SHAM-9× UNX-9× SHAM-1× UNX-1× SHAM-9× UNX-9× SHAM-1× UNX-1×</td>
<td>SHAM-9× UNX-9× SHAM-1× UNX-1×</td>
<td></td>
</tr>
<tr>
<td>Cortex: Tubuloepithelial degeneration/necrosis</td>
<td>a) 4/8 8/8 2/8 3/8 2/8 5/8 1/8 4/8 0/7 0/8 0/8 0/4 0/4</td>
<td>b) (1) (1–2) (1) (1–2) (1) (1) (1) (1) – – – – –</td>
<td>c) 0.5 1.3 0.3 0.5 0.3 0.6 0.1 0.5 – – – – –</td>
</tr>
<tr>
<td>Medulla: Tubuloepithelial swelling</td>
<td>a) 8/8 8/8 0/8 0/8 4/8 4/8 0/8 0/8 0/7 0/8 0/8 0/4 0/4</td>
<td>b) (1–3) (1–2) – – (1–2) (1) – – – – – –</td>
<td>c) 2.3 1.9 – – 0.6 0.5 – – – – – –</td>
</tr>
<tr>
<td>Cortex: Fibrosis</td>
<td>a) 0/8 2/8 0/8 0/8 1/8 5/8 0/8 0/8 0/7 1/8 1/8 0/4 0/4</td>
<td>b) – (1–2) – – (1) (1–2) – (1) – (1) (1) – –</td>
<td>c) 0.4 – – 0.1 0.8 – – 0.1 – – – –</td>
</tr>
<tr>
<td>Cortex: Basophilic tubules</td>
<td>a) 4/8 2/8 0/8 1/8 2/8 4/8 1/8 4/8 1/7 4/8 3/8 0/4 0/4</td>
<td>b) (1) (1–3) – (1) (1) (1–3) (1) (1) (1) (1) –</td>
<td>c) 0.5 0.5 – 0.1 0.3 0.8 0.1 0.5 0.1 0.5 0.4 – –</td>
</tr>
<tr>
<td>Cortex: Mononuclear cell infiltrates</td>
<td>a) 6/8 5/8 2/8 3/8 5/8 5/8 0/8 4/8 0/7 6/8 5/8 0/4 0/4</td>
<td>b) (1) (1–2) (1) (1) (1) (1–2) – (1) – (1) (1) –</td>
<td>c) 0.8 0.8 0.3 0.4 0.6 0.8 – 0.5 – 0.8 0.6 – –</td>
</tr>
<tr>
<td>Cortex: Tubular dilatation</td>
<td>a) 3/8 3/8 0/8 3/8 4/8 4/8 0/8 2/8 4/7 2/8 1/8 0/4 0/4</td>
<td>b) (1) (1–3) – (1) (1) (1–3) – (1) – (1) (1) –</td>
<td>c) 0.4 0.6 – 0.4 0.5 1.0 – 0.3 0.6 0.3 0.1 – –</td>
</tr>
<tr>
<td>Cortex/medulla: Hyaline casts</td>
<td>a) 3/8 3/8 0/8 3/8 4/8 4/8 0/8 2/8 4/7 2/8 1/8 0/4 0/4</td>
<td>b) (1) (1–3) – (1) (1) (1–3) – (1) (1) (1) (1) –</td>
<td>c) 0.4 0.6 – 0.4 0.5 1.0 – 0.3 0.6 0.3 0.1 – –</td>
</tr>
</tbody>
</table>

a) Number of affected animals per number in group. b) Range of severity. c) Group mean severity. – = absent.
ongoing treatment. Zoledronate treatment seems to induce less acute tubular toxicity, but the toxic effect on the tubular cells persists over a longer time with a later peak of α-GST increase.

In general, the time of recovery, indicated by a decrease of α-GST, is delayed in UNX-rats compared with animals with normal renal function.

These findings corroborate the histological changes. Lesions considered to be clearly related to bisphosphonate toxicity were cortical tubulopithelial degeneration/necrosis and multifocal medullary tubulopithelial swelling [3]. Although generally of a minor degree, these were more pronounced in ibandronate treated animals, when compared to zoledronate treated animals, and in UNX-animals compared to SHAM-animals. They were not seen in placebo treated animals, neither in UNX-animals nor in SHAM-animals.

UNX-groups with 9× treatment with either ibandronate or zoledronate showed also some other histopathological changes at a higher degree than those observed in controls: cortical basophilic dironate showed also some other histopathological changes at a placebo treated animals, neither in UNX-animals nor in SHAM-UNX-animals compared to SHAM-animals. They were not seen in treated group with the corresponding 1×

Table 4: Statistical evaluation of the histopathological changes. Compared were every 9× treated group with the corresponding 1× treated group and the corresponding placebo treated group.

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>Tubular epithelial cell degeneration/necrosis</th>
<th>Medullar tubular epithelial cell swelling</th>
<th>Cortical fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNX-IBN-9×</td>
<td>UNX-IBN-1×</td>
<td></td>
<td></td>
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<tr>
<td>SHAM-IBN-9×</td>
<td>SHAM-IBN-1×</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UNX-PL-9×</td>
<td>UNX-PL-9×</td>
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<tr>
<td>SHAM-PL-9×</td>
<td>SHAM-PL-1×</td>
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<tr>
<td>UNX-ZOL-9×</td>
<td>UNX-ZOL-1×</td>
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<tr>
<td>SHAM-ZOL-9×</td>
<td>SHAM-ZOL-1×</td>
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<tr>
<td>UNX-ZOL-9×</td>
<td>UNX-PL-9×</td>
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<tr>
<td>SHAM-ZOL-9×</td>
<td>SHAM-PL-9×</td>
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</table>

n.s. ** *** p ≤ 0.05. p ≤ 0.01. p < 0.001. p < 0.05.

**Acknowledgement**

This study was supported by an unrestricted grant of Roche Pharma, Basel, Switzerland.

**References**

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