Inhibitory Effect of Ouabain on Resistance to Imatinib in Chronic Myeloid Leukemia K562/G01 Cells

CHANG SUN¹, J. LIU², SHIQI ZHAII, H. LIU², L. PENG² AND JING HU*³

¹First Clinical College, Chongqing Medical University, ²College of Stomatology, Chongqing Medical University, ³Faculty of Laboratorial Medicine, Key Laboratory of Medical Diagnostics Designated by the Ministry of Education, Chongqing Medical University, Chongqing 400016, China

Ouabain has shown powerful anti-proliferation activities in various cancers, but its effect on imatinib-resistant chronic myeloid leukemia and toxicity on normal mice has not been investigated. Cell Counting Kit-8 assay was used to detect cytotoxicity and reversal effect of ouabain with different concentration (0.01 µM, 0.1 µM, 1.0 µM, 10 µM) on drug resistance of imatinib-resistant cell line of chronic myeloid leukemia (K562/G01 cell line). Flow cytometry was used to detect the apoptosis effect and cell cycle arrest. Hematological examination, biochemical examination and histological examination were used to detect sub-chronic toxicity of ouabain on healthy mice. In our present study, ouabain showed greatly inhibitory effect and significantly reduced half minimal inhibitory concentration of imatinib in K562/G01 cells, an imatinib-resistant cell line of chronic myeloid leukemia, in a dose- and time-dependent manner, which implied that ouabain increased cell sensitivity to imatinib. Ouabain enhanced apoptosis induced by imatinib in K562/G01 cells not through cell cycle arrest. Animal experiments showed that there were no significant variances in hematological, liver function, kidney function parameters and organ histopathology of all mice groups. These data suggested that ouabain could be a potential agent to treat imatinib-resistant chronic myeloid leukemia for its powerful cytotoxicity as well as reversal effect, but further study is needed to find out its specific mechanism.

Key words: Chronic myeloid leukemia, ouabain, imatinib-resistance, toxicity

Chronic Myeloid Leukemia (CML) is a clonal hematopoietic stem cell malignant tumor featured by Philadelphia chromosome, with an estimate of 34 179 cases and 24 054 deaths reported in 2017¹. BCR-ABL1, a tyrosine kinase with aberration is identified as the key driver in CML, resulting from the fusion of the regulatory regions of BCR and the enzymatic regions of ABL1². Although Tyrosine Kinase Inhibitors (TKIs) such as Imatinib Mesylate (IM) bring a remarkable improvement on the survival rate of early-stage patients, about 40 % of patients with chronic phase have to give up IM because of failure and/or intolerance³, and the drug resistance associated with IM can’t prevent the recurrence and progression of the disease, which has become one of the most important causes of death in patients with CML. Therefore, new drugs that are capable of reversing IM resistance are particularly needed.

Ouabain (fig. 1a) is a rapid-acting Cardiac Glycoside (CG) obtained from seeds of Strophanthus gratus⁴. The controversy over the safety of CGs has existed for a long period chiefly because of its narrow therapeutic window and cardiac toxicity in clinical research⁵, but the clinical security of ouabain is less studied. Ouabain increases intracellular Ca²⁺ concentration by inhibiting Na⁺/K⁺-ATPase, leading to the improvement of myocardial contractility. In addition to its traditional effects, recent researches have proved that ouabain possess abundant health-promoting properties such as anti-inflammatory effect⁶,⁷, anti-coronavirus effect⁸, anti-cancer effect⁹,¹⁰, and reversal effect on drug resistance¹¹,¹².
Ouabain was previously reported to be able to target human leukemia\cite{13}, including strongly interfering intracellular redox homeostasis in lymphocytic leukemia cells\cite{14} and regulating activities of immune cells \textit{in vivo}\cite{15}. Nevertheless, its specific effect on imatinib-resistant CML has not been detected.

In the present study, we explored the effect of ouabain in CML imatinib-resistant K562/G01 cells, an IM-resistant cell line of CML, as well as its possible mechanisms, and explored its toxic effect on normal mice, in order to provide more choices for clinical therapy.

MATERIALS AND METHODS

Preparation of drug stock solution:

The stock solution of IM (Sigma, USA) was prepared by dissolving 10 mg IM in 1.6958 ml Dimethyl Sulfoxide (DMSO) (Solarbio, China) and 15.2622 ml triple steamed water to give 1 mM of stock solution. Ouabain was dissolved in DMSO and prepared into stock solution with a concentration of 10 mM. After filtration and sterilization, store them at -20°.

Cell lines and cell culture:

K562/G01 cells, preserved by our laboratory (Chongqing, China), were cultured in Roswell Park Memorial Institute-1640 medium (Gibco, U.S.A.) containing 10 % fetal bovine serum (Gibco, U.S.A.), 100 µg/ml streptomycin (Hyclone, U.S.A.) and 100 U/ml penicillin (Hyclone, U.S.A.), and placed in 37°, 5 % CO₂ as well as saturated humidity incubator. Cells growing well and in the logarithmic growth period were selected for experiments.

Cytotoxicity assay:

About 5×10³ cells per well were seeded in 96-well plates and incubated with different concentrations of ouabain or IM for 24 h or 48 h and the control group was treated with an equal amount of medium. 10 µl Cell Counting Kit-8 (CCK-8) solution (Taosu, China) was added into each well and the plates were incubated at 37° for another 3 h. The absorbance was measured at the wavelength of 450 nm. Calculated the cell variety (%)=[(OD sample–OD blank)/(OD control–OD sample)]×100 %. The IC₅₀ of IM was estimated by Prism 5 (GraphPad Software, U.S.A). The drug resistance Reversal Fold (RF) values were calculated by dividing the IC₅₀ with IM alone by those with IM and ouabain in K562/G01 cells.

Fig. 1: The effect of ouabain and combination with IM on the proliferation of K562/G01 cells Note: (a): Chemical structure of ouabain; (b): Concentration response curve of K562/G01 cell line derived from CCK-8 cytotoxicity assay performed after 24 h and 48 h exposures with ouabain; (c): Effect of ouabain in combination with IM on proliferation of K562/G01 cells derived from CCK-8 cytotoxicity assay performed after 24 h; Data are presented as mean±SD (n=3), ***p<0.001, compared with control group or 10 µM IM group
Cell apoptosis assay by flow cytometry:

About $5 \times 10^5$ cells per well were seeded in 6-well plates, and cultured with IM (10 µM) or ouabain (0.5 µM, 1 µM) or combination of both medicines. Cells were washed twice with Phosphate Buffered Saline (PBS) after 24 h, and incubated with 5 µl of Annexin-V Fluorescein Isothiocyanate (FITC) labeling solution as well as Propidiumiodide (PI) for 15 min as manufacturer’s instruction. The flow cytometry was used to analyze cell apoptosis.

Cell cycle assay by flow cytometry:

About $5 \times 10^5$ cells per well were seeded in 6-well plates, and cultured with IM (10 µM) or ouabain (0.5 µM, 1 µM) or combination of both medicines. After 24 h, cells were washed twice with PBS and then the cell suspension was added into 75 % cold ethanol and stored at 4° overnight. After cells were washed with PBS, RNase as well as PI stain solution was added. The samples were tested by flow cytometry.

Experimental animals:

Healthy Kunming (KM) mice (n=24), 6 w old, 18-22 g in weight, specific pathogen free grade, with certificate No. SCXK (YU) 2018-0003, were purchased and kept at the Experimental Animal Center of Chongqing Medical University. Mice were reared in cages according to gender (3 per cage) and adapted for 1 w. Room temperature and humidity were strictly controlled and food and acidified water were freely available. All procedures of animals involved in this experiment were in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals and were approved by the Animal Ethics Committee of Chongqing Medical University.

Animal grouping and treatment:

Mice (n=12 female; 12 male) were randomly divided into four groups (n=3/sex/group), high-dose group (1.0 mg/kg ouabain), medium-dose group (0.5 mg/kg ouabain), low-dose group (0.1 mg/kg ouabain) and control group (given an equal amount of saline). Mice were injected (i.p.) every other day for 30 d. After administration, mice were anaesthetized and hematological examination, biochemical examination and histological examination were taken to detect toxicity of ouabain on normal mice.

Observation of general physical signs of mice:

Observed and recorded the daily activities, mental states, food intakes and defeation of mice every day, and weighed them every 2 d, expressing it as weight ratio (weight ratio=weight/initial weight).

Routine blood test:

After administration, blood was adopted by cutting the tails and mixed with the dilution liquid to measure erythrocytes, Mean Corpuscular Hemoglobin (MCH), leukocytes, Mean Corpuscular Volume (MCV), MCH Concentration (MCHC), hemoglobin and Platelet (PLT) with an automatic blood analyzer.

Alanine Aminotransferase (ALT) and Creatinine (CREA) test:

After administration, serum of mice was collected by centrifugation at 3000 r/min for 20 min, and the ALT and CREA were analyzed with kits (Jiancheng, China).

Relative organ/body weight ratio and histological examination:

After euthanasia, necropsy was performed immediately. The heart, liver, kidney, lung and spleen were harvested, and were expressed as relative organ/body weight ratio after measuring. Organs were fixed in 4 % paraformaldehyde for 24 h, then dehydration, paraffin embedding and slicing were performed. Finally, Paraffin-embedded slices were stained with hematoxylin and eosin for further histological examination.

Statistical analysis:

All data were tested by IBM SPSS 26.0 software and represented by mean±Standard Deviation (SD). Comparisons between two groups were performed by Student-t test while comparisons between multiple groups were one-way analysis of variance. p<0.05 was defined as statistical significance.

RESULTS AND DISCUSSION

We estimated the cytotoxicity of ouabain to K562/G01 cells by performing CCK-8 assays. After cells were treated with ouabain at concentrations of 0.01, 0.1, 1, 10 µM respectively for 24 and 48 h, the results confirmed that ouabain obviously decreased the proliferation of K562/G01 cells in
a dose- and time-dependent manner (fig. 1b).

In order to assess the effect of ouabain on IM-sensitivity of K562/G01 cells, cells were treated with IM alone or combination for 24 h, then we tested cell proliferation by CCK-8 assays. The combination of ouabain with 1 µM and IM showed significant difference on cell viability as compared with group only treated with IM alone, revealing that high concentration of ouabain could definitely promote sensitivity of IM to K562/G01 cells, while inhibitory effect on growth of cells which were incubated with IM and ouabain at the concentration of 0.5 µM revealed no statistically significance (fig. 1c). Besides, the IC$_{50}$ of IM in the K562/G01 cells decreased with increasing concentration of ouabain as is shown in Table 1. Ouabain greatly reduced the IC$_{50}$ of IM in K562/G01 cells from 94.92 to 69.56 µM at the lowest concentration (0.01 µM), allowing RF value to achieve 1.36. Moreover, a 131.83-fold increase was induced when cells were treated with 10 µM ouabain. These results demonstrated that ouabain was potential to reverse IM resistance in K562/G01 cells.

In order to assess effect of ouabain in combination with IM on apoptosis of K562/G01 cells, cells were treated with single drug or combination for 24 h, then tested cell apoptosis by Annexin V-FITC/PI double staining. Our flow cytometry results demonstrated that ouabain potentiated IM-induced apoptosis. As shown in fig. 2a, 0.5 or 1 µM ouabain or 10 µM IM slightly enhanced the apoptosis of K562/G01 cells with the rates of 9.94±4.18 %, 17.97±2.2 %, 8.64±2.59 %, respectively. In contrast, the apoptotic percent was 20.11±0.16 % and 25.81±1.4 % when 10 µM IM was combined with 0.5 or 1 µM ouabain. In order to identify if the growth inhibitory effect of ouabain was triggered by cell cycle dysfunction, cell cycle distribution was detected by the PI-based technique. We found that the cells in G$_s$/G$_{0}$, S or G$_s$/M phase had no statistically difference whether treated with single drug or combination, even though the G$_s$/M phase increased in experimental groups (fig. 2b).

It was observed that the daily activities, mental states, food intakes and defecation of mice in all groups were normal during the medication. At concentration of 0.5 mg/kg, weight of both female and male mice showed maximum increment, while growth of those that handled with 0.1 mg/kg ouabain seemed to be the slowest (fig. 3). However, none of the treatment groups showed statistically difference versus control group (p>0.05) (Table 2), and the growth trend of all groups was generally uniform (fig. 3). Blood tests were conducted after administration. In terms of the routine blood test, all of items of mice that were administrated with ouabain at low (0.1 mg/kg), middle (0.5 mg/kg) and high dose (1.0 mg/kg), were little different from those of control group (p>0.05) (fig. 4). ALT and creatinine CREA, which are sensitive liver and kidney damage markers respectively, were used to estimate effects of ouabain on liver function and kidney function of KM mice. Whether ALT or CREA, there were no evident variances between treatment and control groups (p>0.05) (fig. 5).

Relative organ/body weight ratio is a common indicator in toxicology study to assess the effect on the organ. The heart/body weight ratio increased when the dosage increased, and that of both middle-dose group (p<0.05) and high-dose group (p<0.01) were much more than control group (fig. 6a), revealing that hearts of those mice may develop hyperemia or hypertrophy.

**TABLE 1: OUABAIN MODULATED K562/G01 CELLS SENSITIVITY**

<table>
<thead>
<tr>
<th>Group</th>
<th>IC$_{50}$ of IM (µM)±SD</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562/G01+0 µM ouabain</td>
<td>94.92±2.20</td>
<td>-</td>
</tr>
<tr>
<td>K562/G01+0.01 µM ouabain</td>
<td>69.56±2.57</td>
<td>1.36</td>
</tr>
<tr>
<td>K562/G01+0.1 µM ouabain</td>
<td>61.08±8.56</td>
<td>1.55</td>
</tr>
<tr>
<td>K562/G01+1.0 µM ouabain</td>
<td>13.32±1.58</td>
<td>7.13</td>
</tr>
<tr>
<td>K562/G01+10 µM ouabain</td>
<td>0.72±0.33</td>
<td>131.83</td>
</tr>
</tbody>
</table>
Fig. 2: The effect of ouabain in combination with IM on apoptosis and cell cycle of K562/G01 cells
Note: (a) Ouabain in combination with IM on apoptosis of K562/G01 cells derived from flow cytometry performed after 24 h; (b): Effect of ouabain in combination with IM on apoptosis of K562/G01 cells derived from flow cytometry performed after 24 h; *p<0.05 compared with 10 µM IM group

![Graphs showing apoptosis and cell cycle distribution.](image)

Fig. 3: The effect of ouabain on weight of normal mice
Note: (a) Weight ratio of male KM mice; (b): Weight ratio of female KM mice; ( ) Control; ( ) 0.1 mg/kg ouabain; ( ) 0.5 mg/kg ouabain and ( ) 1.0 mg/kg ouabain

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>Sex</th>
<th>Control group</th>
<th>0.1 mg/kg Ouabain</th>
<th>0.5 mg/kg Ouabain</th>
<th>1.0 mg/kg Ouabain</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 d</td>
<td>female</td>
<td>30.70±0.53</td>
<td>29.13±1.56</td>
<td>33.63±2.05</td>
<td>29.63±0.32</td>
</tr>
<tr>
<td></td>
<td>male</td>
<td>32.30±1.25</td>
<td>32.60±0.10</td>
<td>31.07±2.31</td>
<td>33.13±1.97</td>
</tr>
<tr>
<td>2 d</td>
<td>female</td>
<td>33.27±1.87</td>
<td>30.03±1.89</td>
<td>34.63±3.26</td>
<td>31.00±2.26</td>
</tr>
<tr>
<td></td>
<td>male</td>
<td>35.13±1.30</td>
<td>34.97±0.12</td>
<td>33.63±2.51</td>
<td>35.07±1.68</td>
</tr>
<tr>
<td>4 d</td>
<td>female</td>
<td>33.93±2.40</td>
<td>30.67±1.16</td>
<td>35.47±2.51</td>
<td>32.40±1.31</td>
</tr>
<tr>
<td></td>
<td>male</td>
<td>35.03±1.66</td>
<td>35.97±0.06</td>
<td>34.93±2.42</td>
<td>36.07±1.59</td>
</tr>
<tr>
<td>6 d</td>
<td>female</td>
<td>34.33±2.66</td>
<td>32.07±1.39</td>
<td>36.43±3.32</td>
<td>33.10±1.23</td>
</tr>
<tr>
<td></td>
<td>male</td>
<td>36.40±1.71</td>
<td>36.37±0.71</td>
<td>35.97±2.11</td>
<td>37.03±1.46</td>
</tr>
<tr>
<td>8 d</td>
<td>female</td>
<td>35.03±3.37</td>
<td>31.78±1.95</td>
<td>36.83±3.10</td>
<td>34.20±1.35</td>
</tr>
<tr>
<td></td>
<td>male</td>
<td>36.60±0.95</td>
<td>37.33±1.00</td>
<td>36.00±1.21</td>
<td>38.13±1.70</td>
</tr>
<tr>
<td>10 d</td>
<td>female</td>
<td>35.93±3.37</td>
<td>32.67±1.70</td>
<td>37.83±2.20</td>
<td>34.93±1.69</td>
</tr>
<tr>
<td></td>
<td>male</td>
<td>36.60±0.95</td>
<td>37.33±1.00</td>
<td>36.00±1.21</td>
<td>38.13±1.70</td>
</tr>
</tbody>
</table>
### Note:
Values are expressed as means±SD (n=6); *p<0.05 compared with control group.

### Fig. 4: The effect of ouabain on routine blood test of normal mice

Note: (a): Leucocytes (WBC); (b): Erythrocytes (RBC); (c): Hemoglobin (Hb); (d): Mean corpuscular volume (MCV); (e): Mean corpuscular hemoglobin (MCH); (f): Mean corpuscular hemoglobin concentration (MCHC); (g): Platelet count (PLT); values are expressed as means±SD (n=6); p>0.05, compared with control group.
Fig. 5: The effect of ouabain on ALT and creatinine (CREA) of normal mice
Note: (a): ALT; (b): Creatinine (CREA); values are expressed as mean±SD (n=6); p>0.05, compared with control group

Fig. 6: The effect of ouabain on organ index of normal mice
Note: (a): Heart index; (b): Liver index; (c): Kidney index; (d): Lung index; (e): Spleen index; Values are expressed as means±SD (n=6); *p<0.05, **p<0.01, compared with control group

Given that the difference of kidney/body weight ratio (p<0.01) and lung/body weight ratio (p<0.05) only showed in low-dose group (fig. 6c and fig. 6d), it may result from experimental error. In comparison with control group, there were no evident abnormal changes in the organs we tested of treat group whether by naked eyes or under microscope (fig. 7 and fig. 8). No edema, inflammatory infiltration, cell degeneration, necrosis and other pathological reactions were found in heart, liver, spleen, kidney and lung of KM mice (fig. 8).
Ouabain was formerly reported to play anti-leukemia efficacy via inducing apoptosis of leukemia cells\textsuperscript{[16]}, impacting redox homeostasis\textsuperscript{[14]} and targeting related signaling pathway\textsuperscript{[17]}, but the role of ouabain in imatinib-resistant CML remains unclear. Therefore, we assessed its cytotoxicity with different concentrations as well as different treatment duration to investigate the therapeutic potential of ouabain against K562/G01 cells. Our CCK-8 data demonstrated that ouabain could inhibit cell viability in a time- and dose-dependent manner and even low concentration (0.01 and 0.1 µM) of ouabain was able to produce cytotoxicity effect on K562/G01 cells, revealing that ouabain was strikingly competent to inhibit the proliferation of K562/G01 cells.

In order to further clarify whether ouabain could increase the sensitivity of drug-resistant cells to IM, we compared proliferation and apoptosis of K562/G01 cells incubated with single drug or combination. Our data depicted that the inhibitory effect of ouabain with high concentration (1 µM) combined with IM was
significantly stronger than the single IM on cell proliferation as well as induction of apoptosis. These results could be connected with the role of ouabain in the regulation of intracellular calcium increase, as the mitochondrial Ca\(^{2+}\) overload was proved to trigger cytochrome c release, resulting in the activation of caspase 9, which took a crucial part in cell apoptosis\(^{[18]}\). Moreover, the RF increased with the increase of concentration, illustrating that ouabain could definitely inhibit IM resistance in K562/G01 cells in a concentration dependent manner.

As reported, ouabain arrested cell cycle at S and G\(_2\)/M phase for hepatocellular carcinoma cells\(^{[19]}\) and melanoma cells\(^{[20]}\), respectively. However, it is worth noting that effect of ouabain against K562/G01 cells was unrelated to cell cycle arrest according to our data, revealing that ouabain increased K562/G01 cells drug sensitivity through other mechanisms probably due to the drug resistance of that cell line.

Previous data exhibited that ouabain could reverse MDR to cisplatin in esophageal carcinoma cells through targeting Wnt/β-catenin and inhibiting the translocation of β-catenin into the nucleus\(^{[12]}\), rather than cell cycle arrest, which has been deemed as one of the key signaling pathways of CML resistance towards TKIs\(^{[21]}\). On the one hand, the over activation of Wnt/β-catenin brings about nuclear accumulation of β-catenin and abnormal expression of downstream target genes, rendering granulocyte-macrophage progenitors to show self-renewal capacity, which isn’t likely to be possessed normally, causing arrant proliferation of CML cells\(^{[22]}\). On the other hand, β-catenin keeps Leukemic Stem Cell (LSC) maintenance and survival, which is recognized as a crucial mechanism that induces TKIs resistance\(^{[21]}\). Therefore, it would be tempting to suppose that the inhibitory effect of ouabain on Wnt/β-catenin signaling pathway may be relevant with the potentiation of IM-induced cytotoxicity and we intend to verify that hypothesis in the subsequent study.

In recent years, ouabain has shown powerful anti-cancer effects, but its toxicity should not be overlooked in extremely complex human body as most of researches mainly focused on cellular mechanism. Our preliminary study on toxicity of ouabain on normal mice showed that as compared with the control group, there were no evident variances in hematological parameters, ALT, CREA, and organ histopathology of experimental groups after administration of drug. Though there was a slight fluctuation of growth trend, it was reasonable to consider that ouabain hardly affected weight of mice, given that different eating habits or other non-drug factors may interfere with ultimate results. However, heart/body weight ratio greatly increased at dose of 1.0 mg/kg, suggesting that high dose of ouabain might exert adverse effect on heart of normal mice. In line with our results, some literatures stated that ouabain could cause serious cardio toxicity including cardiomyocytes apoptosis\(^{[23,24]}\) and cardiac remodeling\(^{[25]}\). This attributed to the induction of spontaneous contractile activity, mitochondrial Ca\(^{2+}\) overload and cardiomyocyte production of Reactive Oxygen Species (ROS)\(^{[24,26]}\).

As for the difference of lung/body weight ratio and kidney/body weight ratio, we supposed that they were data deviations in given the limited number of mice and normality of histological examination. Therefore, more experimental samples are required to be included to make more accurate conclusions in further studies. Ouabain was also reported to destroy Spiral Ganglion Neurons (SGNs) in rats, gerbils and mice\(^{[27]}\), while it could protect neurons in nanomolar concentration\(^{[28]}\) via modulating intracellular Ca\(^{2+}\) level and signal pathways including mTOR pathway\(^{[29]}\), Wnt/β-catenin pathway\(^{[30]}\) and apoptosis pathways\(^{[31]}\).

Furthermore, another research illustrated that ouabain didn’t exert significant toxicity neither on CD34 cells from healthy people nor on AML mice as estimated by weight loss and mortality\(^{[17]}\). Considering the dual effects of ouabain vary substantially across dose and duration of drug treatment, the exact dosage range also needs to be pinned down, which could be the premise of clinical treatment. Anyway, our pre-clinical research suggested that ouabain only produce a degree of cardiac toxicity in a dose dependent manner and might be a safe drug to healthy mice in general.

In conclusion, our results demonstrated the anti-proliferation effect of ouabain on K562/G01 cells and ouabain in combination with IM results in more remarkable growth inhibition than did IM alone on K562/G01 cells, which
pointed to ouabain as a potential agent to increase the sensitivity of drug-resistant cells to IM. Moreover, long-term application of ouabain did not show obvious toxicity on normal mice generally. Nevertheless, further studies are required to clarify its exact mechanism on TKIs resistance as well as clinically appropriate dose.

Author Contributions:
Chang Sun, J. Liu and Shiqi Zhai contributed equally to this work.

Acknowledgements:
This study was supported by the Natural Science Foundation of Chongqing (CSTB2022NSCQ-MSX0764), and the College Students Innovative Experimental Program of Chongqing Medical University (SRIEP202010).

Conflict of interests:
The authors declare that they have no competing interest.

REFERENCES
28. Kinoshita PF, Orellana AM, Nakao VW, de Souza Port’s NM,

