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Tunicamycin Attenuates Cardiac Automaticity by Reducing HCN Current

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Abstract: *Tunicamycin (TM), as a natural inhibitor of N-linked glycosylation in eukaryotes, can promote apoptosis and sensitize cancer cells to chemotherapy and radiation therapy in numerous studies. The cardiotoxicity of anticarcinogen always deserved serious consideration. Although the promising anticancer effect of TM is exhilarating, more cardiac adverse reactions are remaining needed to identify and explore. Herein, we focus on the electrophysiological aberration after TM application, and unforeseen found that sinus bradycardia is almost the one and only arrhythmia induced by TM. It implies that the impairment of cardiac pacemaker function should be noticed in the adverse cardiac effect of TM. Furthermore, we found that the TM treatment significantly reduced neonatal cardiomyocytes' automaticity, and diminished. If regulating the cardiac automatic action potential. The current findings illustrate the TM antiarrhythmic mechanism, which might be useful for developing N-glycosylation inhibitor anticarcinogen.*

Keywords: *Tunicamycin, N-glycosylation, Bradycardia, HCN channel*

1. Introduction

Tunicamycin (TM) that first isolated from the fermentation broths of Streptomyces lysosuperficus and Streptomyces chartreusis is a natural inhibitor of GlcNAc Phosphotransferase (GPT) in eukaryotes. The N-glycosylation is a major subclass of Glycosylation, which is one prevailing post-translational modification and approximately modulates half of all proteins typically expressed in a cell. More and more studies confirmed that N-glycosylation plays considerable roles in tumorigenesis, cognition, colonization and progression of multitudinous cancers[1-10]. On account of GPT is responsible for the first N-acetylglucosamination of asparagine (N)-linked glycosylation (N-glycosylation) in the endothelial reticulum, so the TM is regarded as a special inhibitor of N-glycosylation [11-13] and supposed as a possible anticarcinogen. Virtually studies have reported that promote apoptosis and sensitize cancer cells to chemotherapy and radiation therapy, such as breast cancer, hypopharyngeal carcinoma, hepatocellular carcinoma, glioblastoma, gastric cancer [2, 14-17]. However, the TM is far from a consummate anticarcinogen, one pivotal obstacle is the toxicity in normal tissues where it is remaining needs more and further study.

The rhythmical beating is the feature of cardiomyocytes, and the various voltage-gated ion channels protein is the characteristic membrane protein in cardiomyocytes. Studies verified TM is able to modify some essential cardiac channel proteins, such as $Ca_V1.2$ channel protein, $Na_V1.5$ channel protein[18, 19]. Considering these ion channels are the basement of the heart rhythm, so the TM may be capable of causing arrhythmias. Meanwhile, the clinical symptoms of congenital disorders of glycosylation patients also hint cardiac abnormality inducing by N-glycosylation defection. 20 percent of congenital disorders of glycosylation patients have been reported to accompany by cardiac complications, such as pericardial effusion, cardiomyopathies, structural defects and arrhythmia[20]. Therefore, the arrhythmia perhaps is

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one of the cardiotoxicities of TM. However, the theoretical cardiotoxicities of TM are still unexamined and unexplored. In this study, we focused on the cardiac electrophysiological adverse effect of TM, and found that TM causes extensive electrophysiological aberration in rats, such as prolongation of RR interval, QT interval, QRS duration. However, it is unanticipated that sinus bradycardia almost is the one and only arrhythmia, although the ECG parameters were widespread altered. Furthermore, we verified TM could attenuate the cardiac automaticity in neonatal cardiomyocytes, and identified the HCN current (funy current, I_f) is impaired during TM application which probably is the noticeable cause of bradycardia.

2. Methods

2.1 Animal and Surgery

Adult Wistar rats (250-300g) were housed for a week at the study site utilizing a 12-h light/dark cycle before surgery. Then a surgery of radio ECG transmitter implantation was performed under sterile conditions. The paired wire electrodes of the transmitters (TA11CTA-F40, Data Sciences International, St.Paul, MN, USA) were placed under the skin of the dorsal and ventral thorax. ECG data recording was set to perform at a sample rate of 10 kHz. The rats would sequentially progress post-surgery recovery period, saline treatment period and TM-treated period. The post-surgery recovery period only contains 3 days' observation without any operations, waiting for the rats recovery from the surgery. During the saline-treated period of 3 days, the rats were applied with 5ml/kg/day saline intraperitoneal injection. And during the TM-treated period, the intraperitoneal injection was continuous, meanwhile, 0.1mg/kg TM was solved in the saline. Heart rate corrected QT interval (QTc) was calculated by standard Bazett's formula, $QTc\text{-}B = QT / (RR)^{1/2}$. The animal treatments were reviewed and approved by the Ethics Committee of the Hebei University of Chinese Medicine.

2.2 Neonatal Cardiomyocytes Culture

Neonatal cardiomyocytes were prepared from 1-3-day-old Wistar rats by an enzymatic method. Hearts were removed from neonatal rats and the ventricles were minced into 1-mm3 pieces in iced buffer solution, then digested 2-3 times with 0.05% collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ, USA) in buffer solution at 37℃ for 10 min. The dispersed cells from each digestion were combined and purified by centrifugation (1500rpm, 3min). Cardiomyocytes were distinguished from cardiac fibroblasts by different attachment properties. Then the isolated cardiomyocytes were cultured in a mixed medium comprising Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum, 100 unit/ml penicillin and 100 μg/ml streptomycin at 37℃ in a 95% O_2 - 5 %CO_2 incubator.

2.3 Electrophysiology

The electrophysiological experiments were performed using the EPC-9 amplifier (HEKA Elektronik, Germany) in neonatal cardiomyocytes in 37℃ maintaining by a heater. The cell-attached current-clamp mode was applied for recording action potentials of neonatal cardiomyocytes, bath solution and pipette solution are same (in mM): $NaCl_2$ 140, KCl 5.4, HEPES 10, $MgCl_2$ 1, glucose 10, $CaCl_2$ 1.8 (pH 7.4 with NaOH). The whole-cell voltage-clamp mode was applied for the hyperpolarization-activated cyclic nucleotide-gated channel current recording. The pipette solution contained: KCl 140, $MgCl_2$ 1, HEPES 5, EGTA 10, Mg-ATP 5 (pH 7.2 with KOH). And the bath solution contained (in mM): NaCl 135, KCl 5.4, $BaCl_2$ 2, $MgCl_2$ 1, 4-AP 0.5, $NiCl_2$ 2, HEPES 10, glucose 10, $CaCl_2$ 1.8 (pH 7.4 with NaOH) solution KCl 140, $MgCl_2$ 1, HEPES 5, EGTA 10, Mg-ATP 5 pH 7.2 with KOH). The hyperpolarizing voltage steps (3s) up to -140 mV were applied from VHP of -40 mV. When identified the cyclic nucleotide sensitivity of I_f, the 10 μM isoproterenol (Daiichi-sankyo, Japan) was added into the bath solution for increasing the intercellular cyclic nucleotide.

2.4 Statistical Analysis

Data were acquired by using computer software (Pachmaster, HEKA), and all curve fittings and figures were made on Sigma Plot ver. 10 (Systat Software Inc, USA). All results are presented as mean ± SE, with the number of observations indicated by n. Values were analyzed by Student's t-test or paired Student's t-test when data were obtained from repeated measurement design. Differences were considered significant at $p<0.05$.

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3. Results

3.1 Tunicamycin Caused Serious Sinus Bradycardia in Rats

We applied intraperitoneally injection with 0.1 mg/kg/day TM to 4 rats and recorded 24 hours ECG of rats.The rats sequentially underwent post-surgery recovery, saline treatment and TM treatment (Fig.1a). The heart rhythm of rats didn't have conspicuous alteration in the first 2-3 days' TM treatment, but the heart rate progressively decreasesd up to serious sinus bradycardia, and died within 5-7 days' TM treatment (Fig.1b). It was noteworthy, the sinus bradycardia was nearly the only arrhythmia induced by TM treatment. Heart rate slows down from 377.8±14.3 beat/min in the saline treatment period to 237.1±12.1 beat/min on the last day with TM treatment (Fig.2a). Other arrhythmias occurred in the stage of impending death stage, all rats ultimately died with serious bradycardia + third-degree A-V block + ventricular escape rhythm (Fig.2). This result implied that the sinus bradycardia was the important cardiotoxic manifestation of TM.

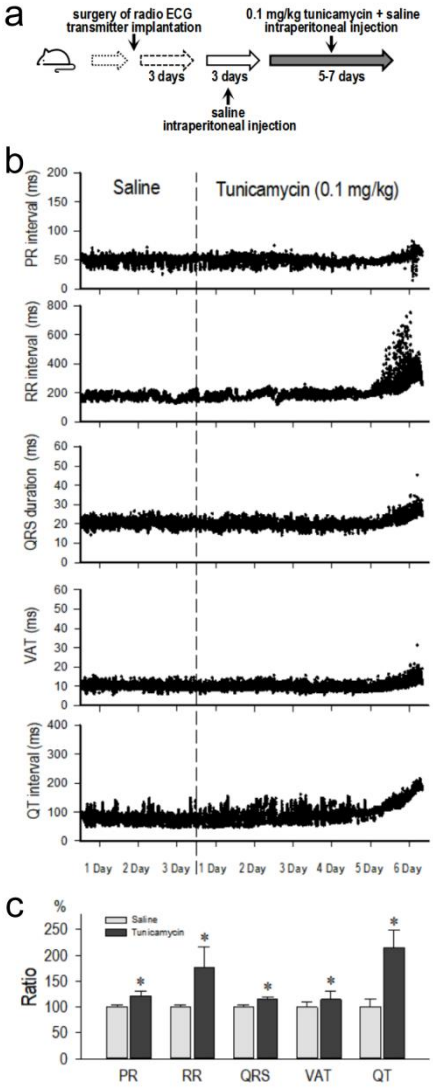

Figure 1: TM treatment widespread deterioration the ECG parameters in rats.

(a) Diagram of an animal treatment protocol. The rats sequentially progressed post-surgery recovery period, saline treatment period and TM-treated period after radio ECG transmitter implantation surgery. (b) Scatter diagrams of ECG parameter data from a representative rat. (c) The comparison of ECG parameters between saline-treated period and last TM-treated day. Values represent the mean±SE. *p<0.01 vs. Vehicle.

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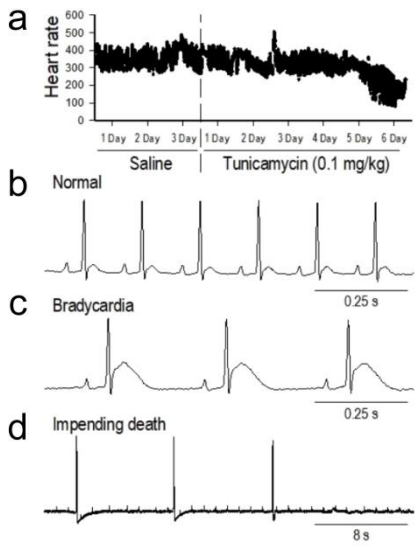

Figure 2: TM treatment caused serious bradycardia rats.

(a) Representative heart rate recording from a representative rat. (b) (c) (d) Representative electrocardiograph of different stage. Values represent the mean ±SE. *p<0.01 vs. Vehicle.

3.2 Alteration of ECG Parameters Induced by Tunicamycin

The parameters of 24 hours ECG were obtained from computer analysis. Fig.1b and c demonstrates the representative recording of 24 hours ECG parameters during saline treatment and TM treatment. During the saline treatment phase, the ECG stays at a regular status. When entering the TM treatment phase, the ECG still kept normal status at the initial 2-3 days, but then all the major parameters swiftly deteriorated. Among the parameters, the RR interval and QT interval have a huge increase (Fig.1c). Comparing saline treatment period, the averaged RR interval of last day with TM treatment has a 1.7-time prolongation, from 160.9 ±6.5 ms to 282.2 ±32 ms, p<0.05, n=4. And the averaged QT interval has a 2.1-time prolongation, from 58.2 ±9.1 ms to 125.3 ±9.7 ms, p<0.01, n=4. Beside, the PR interval, QRS duration also have a modest alteration, from 48.3 ±1.8 ms to 58.7 ±2.1 ms and from 19.1 ±0.7 ms to 22.0 ±2.3 ms (Fig.1c).

Heart rate corrected QT interval (QTc) is a more exact index in the assessment of repolarization changes considering the safety of drugs and cardiac disorders than QT. Consider the lower heart rate after TM-treatment, We calculated the QTc by standard Bazett's formula[21] The averaged QTc of 24 hours before death grows significantly escalate than of saline treatment period, from 144.6 ±19.8 ms to 233.3 ±15.3 ms, p<0.001, n=4. The QTc prolongation may contribute to the generation of ventricular tachycardia in the rats, but all rats died with sinus bradycardia, not ventricular tachycardia, hence we presume heart rate reduction is more critical than QTc prolongation.

3.3 TM Treatment Reduced Cellular Automaticity in Neonatal Cardiomyocytes

Afterward, TM weakening automaticity was reconfirmed in cardiomyocytes. The monophasic action potential recordings of neonatal cardiomyocytes incubated with or without TM are exhibited in Fig.3. After 24 hours 10 mg/L TM application, the cells' automatic beating frequency presented a significant reduction, from 98.80 ±7.11 BPM to 42.20 ±9.72 BPM, p<0.01. The reduction of cellular automaticity reconfirmed the bradycardia caused by TM in rats. Collectively, the N-glycosylation inhibition by TM is able to attenuate cardiac automaticity in vivo and in vitro, so the impairment of cardiac pacemaker function should be noticed in the cardiac adverse effect of TM.

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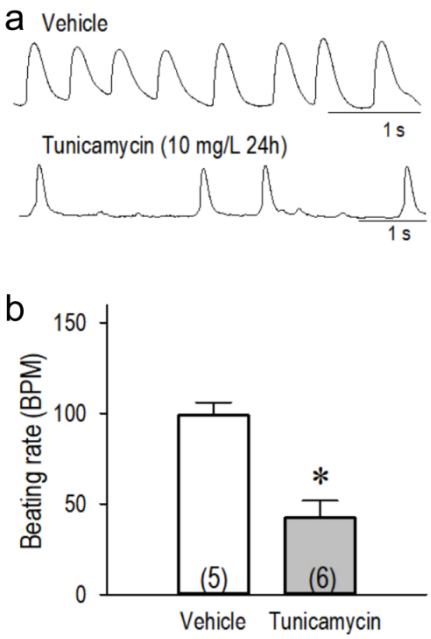

Figure 3: The TM treatment attenuated the cellular automaticity of neonatal cardiomyocytes.

(a) Representative action potentials recording of neonatal cardiomyocytes applied with or without TM. (b) Histogram of beating rate frequencies. After 24 hours of 10 mg/L TM application, neonatal cardiomyocytes' automaticity showed an apparent reduction, from 98.80±7.11 BPM to 42.20±9.72 BPM, $p<0.01$. Values represent the mean±SE. *$p<0.01$ vs. Vehicle.

3.4 Tunicamycin Attenuated the If in Neonatal Cardiomyocytes

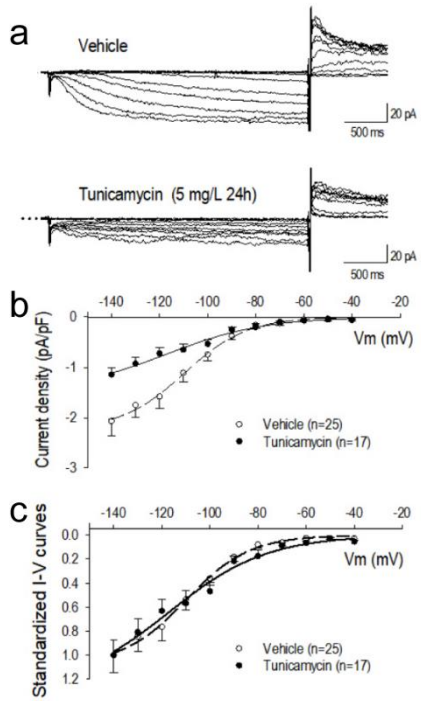

Figure 4: TM treatment attenuated the I_f in the neonatal cardiomyocytes but not change the gated properties of the I_f.

(a) Representative current traces of I_f with or without TM application. (b) The I-V curves of I_f. Under 24 hours 5 mg/L TM-treatment, I_f had a significant reduction. The $I_{f,-140mV}$ reduced from -2.07±0.29 pA/pF (n=25) to -1.15±0.14 pA/pF (n=17), $p<0.05$. (c) The standardized I-V curves of I_f. Values represent the

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mean±SE. *p<0.01 vs. Vehicle.

The voltage-gated ion channel currents are the basis of cardiac electric activities. So, we speculated the impaired ion channel current is one important cause of the bradycardia caused by TM. It's well known that the I_f generated by hyperpolarization-activated cyclic nucleotide-gated channel (HCN channel) is responsible for the major inward current during the spontaneous repolarization phase 4 of the action potential, and hence is a crucial regulator of cardiac automaticity. In the present study, we examined the effect of TM on I_f in neonatal cardiomyocytes. When 10 mg/L TM was applied, the I_f was too tiny to detectable by whole-cell patch-clamp. Then TM was decreased to 5 mg/L, the I_f presented an approximate 50% reduction (Fig.4a and b), the I_f, -140mV reduced from -2.07±0.29 pA/pF (n=25) to -1.15±0.14 pA/pF (n=17), p<0.05. Considered the pivotal role of I_f in the cardiac automaticity, the I_f reduction was a noticeable reason for the sinus bradycardia caused by TM. However, there was no significant difference between the standardized I-V curves with or without TM, so the voltage-dependent property of I_f was not altered by TM(Fig.4c).

3.5 Tunicamycin Did Not Alter the Gating Properties of the HCN Channel

HCN channel is denominated according to its hyperpolarization-activation and cyclic nucleotide sensitivity. The intercellular cyclic nucleotide sensitivity enables I_f had key roles in the positive chronotropic effect of catecholamine, and also was examined in the present study. After added 10 μM isoproterenol into the bath solution, the I_f was remarkable enlarged whether with or without TM (Fig.5a and b) . After standardization, we found that the growth ratios of I_f were statistically identical between TM group and vehicle group (Fig.5c), which suggested that TM also not regulated the cyclic nucleotide sensitivity of I_f. So, TM seemly only reduced the amplitude of I_f, without changing the properties.

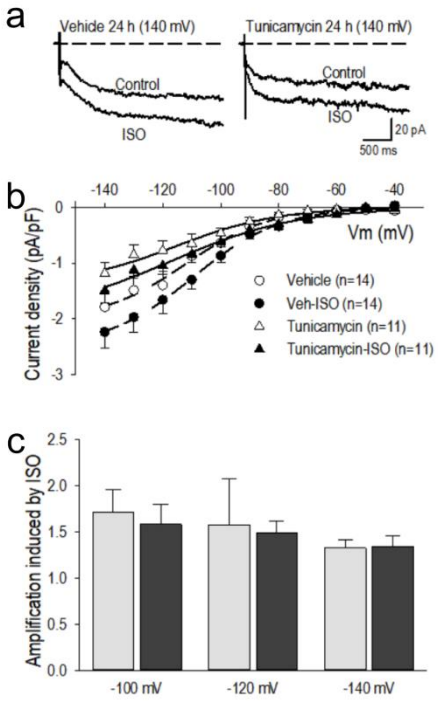

Figure 5: TM treatment did not change the sensitivity of cyclic nucleotides of the I_f.

(a) Representative current traces of $I_{f,-140mV}$ before and after 10 μM isoproterenol stimulation. (b) The I-V curves of I_f before and after isoproterenol stimulation. (c) The growth ratios of I_f under different hyperpolarizing voltage. Values represent the mean±SE. *p<0.01 vs. Vehicle.

4. Discussion

N-glycosylation inhibitor is a novel trend of anticarcinogen[10]. The N-glycosylation is denominated from that the glycans are attached to the nitrogen atom of an asparagine (N) which is present as a part of Asparagine-X-Serine/Threonine-X (N-X-S/T-X) consensus sequence. The N-glycans, due to their diverse sugar molecules arrangement, provide a great structural and functional variety to the protein that

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is necessary for biological processes. But sometimes the excessive N-glycosylation will contribute to some malignant processes. At present, aberrant glycosylation has been widely recognized as an important hallmark of cancer and significantly correlates with the development, progression, metastasis and chemoresistance of tumors[1-10]. Therefore the intervention of N-glycosylation is considered to apply in anticancer therapy. TM is a canonical compound for blocking N-linked glycosylation by inhibiting the transfer of UDP-N-acetylglucosamine (GlcNAc) to dolichol phosphate in the endoplasmic reticulum (ER) of eukaryotic cells, thus disrupting protein maturation[11-13]. TM, as a natural N-glycosylation inhibitor, was consequently tried in anticancer therapy. Inspiringly, a number of studies have confirmed that TM could sensitize cancer cells to chemotherapy and radiation therapy, including multidrug-resistant gastric cancer cells, hepatocellular carcinoma, breast cancer cells, and has been identified as a promising anticancer therapeutic[2, 16, 22-25]. However, TM is far from a consummate anticarcinogen, one pivotal obstacle is the toxicity in normal tissues.

The cardiotoxicity of anticarcinogen always deserved serious consideration. An increasing number of oncology patients are facing cancer therapy-related cardiac dysfunction risk, leading to the emerging field of cardio-oncology (also known as onco-cardiology)[26]. Although the excellent anticancer effect of TM is exhilarating, more cardiac adverse reactions are remaining need to identify and explore. In this study, we inspected the cardiac electrophysiological abnormalities caused by TM, and unforeseen found the cardiac automaticity of rats is susceptibly impaired after 3 day's 0.1 mg/kg/day TM intraperitoneal injection, and all rats were dead within 7 days. The TM tolerance may differ from species and potency, frequency of TM application. The nude mouse is enough to withstand 4 weeks 0.5 mg/kg/week TM intraperitoneal injection, and the high dose TM-treatment exhibit appreciable anticancer effect in nude mouse xenograft models of human hepatocellular carcinoma[15]. In comparison, a single 1 mg/kg dose of TM is sufficient to cause serious inflammation and necrosis in rat livers[27].

On account of N-glycosylation exists in massive protein, the TM wildly regulates numerous biological processes. For example, TM sensitized hepatocellular carcinoma to cisplatin-induced apoptosis and reversed drug resistance by regulating the DPAGT1/Akt/ABCG2 pathway[16]. And cotreatment with TM and Adriamycin dramatically decreased the viability of gastric cancer cells, especially multidrug-resistant cells, by triggering ER stress-associated apoptosis[2]. While as for the cardiac electrophysiological abnormalities caused by TM in the present study, the N-glycans deficiency of voltage-gated ion channels perhaps an appropriate reason. It was reported that multiple cardiac ion channels, including $Na_V1.5$, $Ca_V1.2$, $K_V11.1$, $K_V1.4$, $Ca_V3.1$ were modulated by N-glycosylation[18, 19, 28, 29], and the arrhythmia also was observed inCDGs and N-glycosylation defect animals[18, 20]. Our result was consistent with the previous studies, TM caused widespread aberration of ECG parameters in rats which indicated diverse impairment of ion channel current. But unanticipated, although ECG parameters were extensively altered, the bradycardia seems to be the only arrhythmia caused by TM. Meanwhile the cardiac automaticity diminution was reconfirmed in neonatal cardiomyocytes by spontaneous action potential recording. So the pacemaker-related ion channels perhaps need more attention in the cardiotoxicity of TM. The I_f generated by HCN channels is the inward currents in the automatic depolarization phase 4 of the action potential of pacemaker cells, which is crucial for the spontaneous electric action of the sinoatrial node[30, 31]. The HCN channel also belonged to N-glycosylation proteins. Although the exact function of N-glycans in the dominant cardiac isoform HCN4 is still unclear, it was reported that mutation of N-glycosylation site of HCN1 and HCN2 ($HCN1_{N327Q}$ and $HCN2_{N380Q}$) led to channel protein fail to arrive at the membrane and hence reduces the I_f [32, 33]. Similar to the previous studies, we also observed the TM treatment significantly diminish I_f, and speculated it was the noticeable reason for the sinus bradycardia caused by TM. Furthermore, we also examined the voltage-gating properties and intercellular cyclic nucleotide sensitivity of I_f, consistent with $HCN1_{N327Q}$ and $HCN2_{N380Q}$, the N-glycosylation deficiency induced by TM application did not alter the gating kinetics of I_f. So, the TM seemly only reduced the amplitude of I_f, without changing the properties.

5. Conclusions

We scrutinized that the cardiac electrical aberration caused by TM in vivo by radio ECG transmitter implantation, and found that the bradycardia was the dominant arrhythmia after application. Then we re-verify TM will diminish cellular automaticity in vitro, and identify the reduction of I_f. is an influential reason. These results contribute to further understanding of the cardiotoxicity of TM, and may promote the discovery and development of N-glycosylation inhibitor anticarcinogen.

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Conflict of interest: The authors declare no conflict of interest.

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