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Effects of Ketamine Combined with Medetomidine or Dexmedetomidine on the Ear Function of Mouse Offsprings

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Abstract: Ketamine combined with Medetomidine (MK) or Dexmedetomidine (DK) is widely used to provide general anaesthesia in laboratory animals. This study aimed to assess the effects of MK or DK treatment on embryogenesis and inner ear development. The 25 pregnant mice were divided into 5 groups and administrated Intraperitoneal (IP) or Subcutaneous (SC) with MK or DK at embryonic day 8.5. After birth, the Pups were counted and weighed at postnatal days 0, 15 and 30. For ear function, Brainstem Response testing (ABR) was applied to check the cochlea and reaching reflex and swimming tests were applied to assess vestibular function. The results showed no apparent difference between MK or DK administered either IP or SC in pregnant mouse. No significant difference was found between treated and control groups in embryo development and ear function. The ABR thresholds of treated groups were also not statistically significantly different from control group ($p>0.05$). Treated Pups exhibited normal reaching reflex and swimming behavior and showed normal morphology in all six inner ear sensory regions. In conclusion, this study did not find ketamine combined with medetomidine or dexmedetomidine had any effect on general embryogenesis and ear function of mouse offsprings.

Key words: Ketamine, medetomidine, dexmedetomidine, ear, mice

INTRODUCTION

Mice are the most common animals used in experimental biology and psychology studies because they share a high degree of homology with humans. The mouse is also one of the most frequently anaesthetized animal in laboratory research (Green, 1979). Ketamine alone (Green *et al*., 1981) or combined with other drugs, such as α-2 agonists (Nevalainen *et al*., 1988; Flecknell, 1996) have been described as a chemical restraint for a variety of experimental protocols in different laboratory animal species. Ketamine is a centrally acting NMDA-receptor antagonist that rapidly induces dissociative anaesthesia while providing analgesia. α_2-agonists inhibit the enzyme Adenylate cyclase which leads to the inactivation of the secondary messenger cyclic adenosine monophosphate and induces smooth muscle and blood vessel constriction. Now, α_2-agonists are commonly used in small animal anaesthesia because their potent sedative and analgesic action contributes to a significant drug sparing effect. When ketamine was combined with an α-agonist, general anaesthesia may be attained.

Ketamine in combination with medetomidine has been widely used as an anaesthetic for laboratory animals (Arras *et al*., 2001; Richardson and Flecknell, 2005; Granholm *et al*., 2006). Medetomidine is an equal mixture of two optical enantiomers, dexmedetomidine and levomedetomidine. The latter is generally considered to be pharmacologically inactive (MacDonald *et al*., 1991). Dexmedetomidine is a highly selective and potent α_2-agonist with 8 times higher affinity for the α_2-adrenoceptor than clonidine. A handful of studies have compared the sedative effects of medetomidine and dexmedetomidine in mouse (Burnside *et al*., 2013), dog (Kuusela *et al*., 2000; Murrell and Hellebrekers, 2005), cat (Granholm *et al*., 2006) and sheep (Biccard *et al*., 2008). Almost all of these studies concluded there were no significant differences between dexmedetomidine and medetomidine in clinical trials. As of today, effects of ketamine combined with medetomidine or dexmedetomidine on offsprings have not been reported.

Normal ear function is important for lab animals, especially in audiology research. The special sense of hearing requires functional integration of the outer, middle and inner ears (Hudspeth, 1997). The vertebrate inner ear which contains the sensory organs for hearing and balance can be easily damaged by drug treatment. In this study, timed pregnant mice were administered by intraperitoneal or subcutaneous (injection) with

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Ketamine+Medetomidine or Ketamine+Dexmedetomidine combinations. Then their offsprings will be measured and the Pup's ear function will be tested.

MATERIALS AND METHODS

Timed pregnant mice breeding and drug treatment: The animal care and use procedures described are approved by the Jilin University Animal Care and Use Committee. The 25 ICR females (8 weeks) were bred with 5 B6 males. The 12 N on the day a vaginal plug was detected was considered embryonic day 0.5 (E0.5). Timed pregnant mice were treated by ketamine (75 mg kg^{-1}) combined with medetomidine (1 mg kg^{-1}) or dexmedetomidine (0.5 mg kg^{-1}) at embryonic day 8.5 (E8.5). The 25 dams were divided into 5 groups (5 dams per group) with different anaesthetic treatments. group I: No administration (control); group II: Ketamine+ Medetomidine with Intraperitoneal a dministration (MK-IP); group III: Ketamine+Dexmedetomidine with intraperitoneal administration (DK-IP); group IV: Ketamine+Medetomidine with Subcutaneous administration (MK-SC); group V: Ketamine+ Dexmedetomidine with Subcutaneous administration (DK-SC).

Weight and number of offsprings: The 25 ICR dams gave birth on E19.5. Number of Pups in each litter were counted. The 40 Pups from 5 L in each group were weighed at postnatal days 1, 15 and 30 (P1, P15, P30).

Auditory brainstem response testing: Auditory thresholds were determined by Auditory Brainstem Response (ABR) testing (Wang *et al*., 2011). The 8 Pups from each group were randomly selected for the ABR test. Adult mice (P30) were anesthetized using a Ketamine/ Xylazine/Acepromazine (K/X/A) cocktail in 0.9% saline (Barkdull *et al*., 2007). A speaker was connected to the ear canal using a flexible tube and three electrodes were inserted under the skin. Tone bursts at 4, 8, 16 and 32 kHz were presented as stimuli. The duration of each tone burst was 2 msec and responses were recorded for 12 msec. Responses were then averaged to obtain an event related potential at each frequency and intensity.

Behavioral assessment of vestibular function: Mice underwent reaching reflex and swimming tests to assess vestibular function. To test the reaching reflex, each mouse was held by its tail and lowered toward a surface. Mice with impaired vestibular function tend to curl toward their tail but normal mice tend to stretch their paws out toward the approaching surface. To test swimming ability, the mice were placed in a clear plastic mouse cage filled with warm water at 30°C. While in the water the mice were observed and analyzed. Normal swimming behavior is defined as follows: after being placed in water, mice managed to keep their noses and tails above the surface of water and swam toward the side of the cage (Goodyear *et al*., 2012).

Cryostat sections and immunohistochemistry: The 3 Pups from control and treated group were euthanized at P0. The whole heads were fixed in 4% PFA overnight at 4°C. The whole inner ear were dissected and cryoprotected in graded sucrose/PBS (10, 20 and 30% until the tissue had sunk) embedded in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC) and serially sectioned at 12 μm in the coronal plane.

All tissues were permeabilized and blocked in 0.2% saponin in blocking solution (PBS containing 10% bovine serum albumin and 10% serum from the species in which the secondary antibody was generated). The hair cell marker, Myosin 7a (dilution 1/100; Proteus Biosciences Inc, Ramona, CA) was applied in blocking solution overnight at 4°C with gentle agitation. Alexa Fluor- conjugated secondary antibody was applied in blocking solution overnight at 4°C. Alexa-conjugated phalloidin (dilution 1/150; Molecular Probes) was applied in PBS for 1 h at 25°C. Sections were mounted in VectaShield and imaged with the Olympus FV1000 laser-scanning confocal microscope.

Statistical analyses: The average ABR thresholds are presented in Fig. 1 as mean dB SPL±Standard Deviation (SD) and compared by t-test to identify statistically significant relationships.

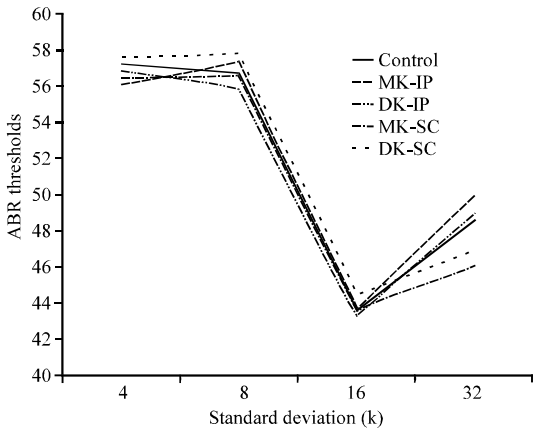

Fig. 1: ABRs results for all treated group at P30. The average ABR thresholds in Sound Pressure Level (dB SPL) as means±Standard Deviation (SD) are plotted for all mice at 4, 8, 16 and 32 kHz. In control group (black line), MK-IP group (red line), DK-IP group (green line), MK-SC group (pink line) and DK-SC group (blue line) groups are not statistically significantly different from each other ($p>0.05$)

Data were analyzed by one-way ANOVA using SPSS (Statistics Production for Service Solution, USA) Software after being transformed via LSD. Significance was taken as $p<0.05$.

RESULTS AND DISCUSSION

All dams treated with different anaesthetic administration gave birth on embryonic day 19.5. The results of Pup's number and weight were shown in Table 1. Around 10 Pups were found from each litter. No difference was found between treated (10.5±2.89, 10.25±3.30, 10.5±2.38, 10.75±2.99) and control (10.25±2.22) groups ($p>0.05$). The 40 Pups from each group were weighed at P1, P15 and P30. The weight increased normally with days. Pups from different treatments (MK-IP, DK-IP, MK-SC, DK-SC) had similar growth rate with control group ($p>0.05$).

As described earlier, ABRs were measured for all mice at P30. The average ABR thresholds in Sound Pressure Level (dB SPL) as mean±Standard Deviation (SD) were plotted for all mice at 4, 8, 16 and 32 kHz. The 16 ears from 8 mice were tested in each group. In the control group, the average ABR thresholds were 57.25±7.59 (for 4 k), 56.75±5.39 (for 8 k), 43.63±7.59 (for 16 k) and 48.62±4.34 (for 32 k) dB SPL (Fig. 1, black line). ABR thresholds of the MK-IP group were slightly higher than that of the control group in 8, 16 and 32 k ($p>0.05$; Fig. 1, red line). ABR thresholds of DK-IP group were slightly lower than that of the control group in 4, 8 and 16 k ($p>0.05$; Fig. 1, green line). The ABR thresholds of MK-SC (Fig. 1, pink line) and DK-SC (Fig. 1, blue line) groups are also not statistically significantly different from that of the control group ($p>0.05$).

For assessment of vestibular function, reaching reflex and swimming behavior of mice were tested. The mice from control and 4 treatment groups were able to exhibit normal swimming behavior with an elongated, balanced posture and tail extending caudally like a corkscrew (Fig. 2A) had no circling or head bobbing behaviors; displayed normal reaching behavior and stretched their paws out toward the approaching surface (Fig. 2B).

Researchers used Immunochemical Method to check the inner ear morphology. Inner ears from mice with different treatment were collected and sectioned as described. Sections were stained with Myo 7a and phalloidin to detect hair cells. Researchers found that the inner ears from MK or DK treated offsprings displayed the similar morphology with the control ones. Some representative results were shown in (Fig. 3). After treatment, organ of corti had one row of inner hair cells and three rows of outer hair cells as per normal conditions (Fig. 3a); crista (Fig. 3b) saccular macula (Fig. 3c) and utricular macula (Fig. 3d) had healthy hair cells and hair bundles.

Table 1: Weight and number of Pups

Treatments	Weight of Pups (P1)	Weight of Pups (P15)	Weight of Pups (P30)	No. of Pups per litter
Control	1.72±0.26[a]	9.10±0.66[a]	26.02±2.45[a]	10.25±2.22[a]
MK-IP	1.75±1.23[a]	9.12±0.37[a]	25.99±2.23[a]	10.05±2.89[a]
DK-IP	1.79±0.29[a]	9.04±0.74[a]	26.35±1.48[a]	10.25±3.30[a]
MK-SC	1.75±0.27[a]	8.89±0.85[a]	26.03±2.16[a]	10.05±2.38[a]
DK-SC	1.77±0.29[a]	8.96±0.78[a]	25.96±2.76[a]	10.75±2.99[a]

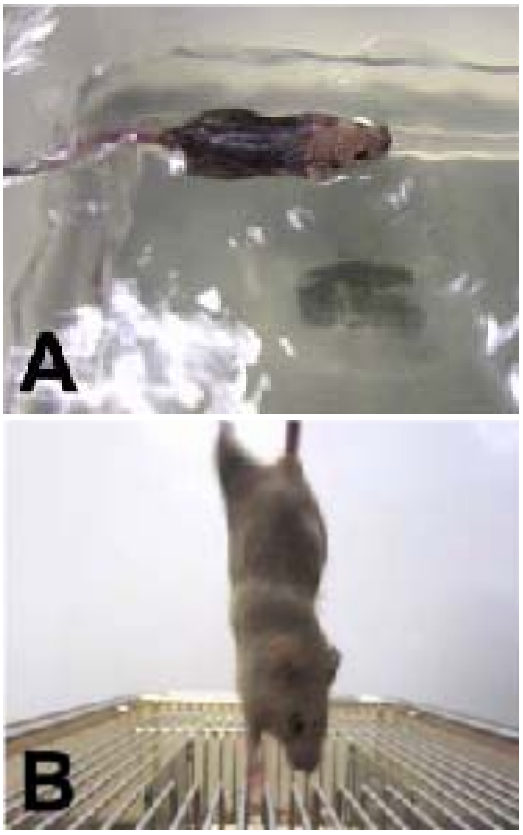

Fig. 2: Assessment of vestibular function; A) the mice from control and 4 treatment groups were able to exhibit normal swimming behavior with an elongated, balanced posture and tail extending caudally and B) displayed normal reaching behavior

The mammalian hearing apparatus consists of three compartments, the outer, middle and inner ears. The outer ear collects acoustic waves from the air which are amplified and transmitted by the middle ear into the inner ear to generate vibrations. There are six distinct sensory organs in the mammalian inner ear: the organ of corti of the cochlea, the two maculae of the saccule and utricle and the three cristae of the semicircular canals. The organ of corti is the organ of hearing. These vibrations excite the hair cells (one row of inner hair cells and three rows of outer hair cells) of the organ of corti located in the

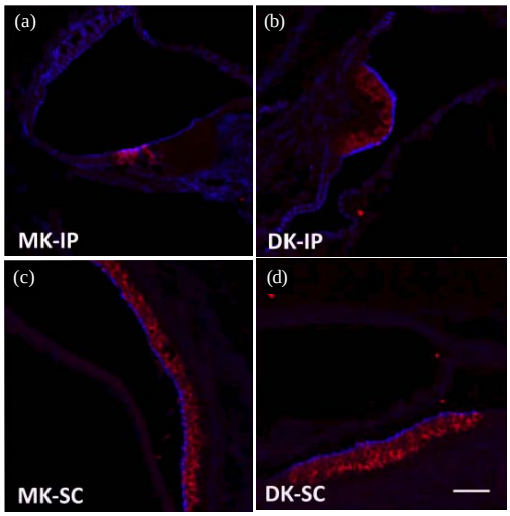

Fig. 3: Morphology of treated inner ear. After treatment, organ of corti had normal one row of inner hair cells and three rows of outer hair cells; a) crista; b) saccular macula; c) utricular macula; d) had healthy hair cells and hair bundles. Scale bar (50 μm) in d applies to all panels

cochlear part of the inner ear to generate neural impulses that are transmitted to the brain through the acoustic nerve. The inner ear is also involved in the process of balance, centered in the vestibular area. The cristae and the maculae are vestibular organs that respond to angular and linear acceleration, respectively. So, ear function is really important for both animals and human beings. Hearing impairment following many types of anesthetic techniques has already been reported previously in human (Hussain *et al*., 1996; Pau *et al*., 2000). And it is reported ketamine, medetomidine and dexmedetomidine could cross the placenta readily and provide anaesthesia and analgesia for the fetus when they are delivered (Ellingson *et al*., 1977; Ala-Kokko *et al*., 1997; Musk *et al*., 2012). However, till now anesthetic effects on ear function of offspring have not been reported. In this study, pregnant mice were treated by ketamine combined with medetomidine or dexmedetomidine. Then, medetomidine and dexmedetomidine were compared and the offspring's ear development and function were studied.

The mouse inner ear initially becomes evident at around 8 days of embryonic development. In E8.5 mouse embryos, the inner ear starts from the otic placode to invaginate to form the otic cup (Hilfer *et al*., 1989) and then otic vesicle or otocyst (Sher, 1971; Marovitz *et al*., 1976; Hilfer *et al*., 1989). In the study, researchers treated the mice at 8 days after mating to test whether those drugs had any affects on inner ear development. From Fig. 3, researchers found that after treatment the inner ear showed normal morphology. Organ of corti had one row of inner hair cells and three rows of outer hair cells as in normal conditions; crista, saccular macula and utricular macula had healthy hair cells and hair bundles. Cochlear function and central auditory transmission were assessed using ABR test. I.p. injections of a K/X/A anaesthetic cocktail were applied for ABR test. As reported, the K/X/A based anaesthetic produced stable ABR thresholds and gain over time (Cederholm *et al*., 2012). ABR results showed that the cochlea function of treated offsprings was as good as the untreated ones. From Fig. 2, the treated mice showed normal vestibular behavior in both reaching and swimming tests. Therefore, after treatment with ketamine combined with medetomidine or dexmedetomidine at E8.5, the offsprings still had normal inner ear in both morphology and function.

α_2-agonists are commonly used in small animal anaesthesia for their potent sedative and analgesic properties. The α_2-agonist medetomidine is an equal mixture of dexmedetomidine and levomedetomidine. Dexmedetomidine is the active part and levomedetomidine is pharmacologically inactive (MacDonald *et al*., 1991). Medetomidine and dexmedeto-midine are routinely administered alone or in combination with other drugs such as ketamine. Till now, no study that compared medetomidine and dexmedetomidine as a sedative or premedication has found clinically significant differences in various species (Kuusela *et al*., 2000; Murrell and Hellebrekers, 2005; Burnside *et al*., 2013). The study failed to demonstrate any advantages of the active enantiomer dexmedetomidine over the mixture medetomidine when combined with ketamine for general anaesthesia in mice. The findings showed no apparent difference between MK or DK administered either IP or SC in pregnant mouse. After MK or DK treatments, embryos could continue to develop normally and be born with normal weight. The ear function of offsprings was as normal as control ones. So, ketamine combined with medetomidine or dexmedetomidine could not affect the ear function of mouse offsprings which will benefit audiology studies.

CONCLUSION

This study did not find that ketamine combined with medetomidine or dexmedetomidine had any effects on general embryogenesis and ear function of mouse offsprings. However, future studies on the effects on other organs and systems of offsprings are still needed.

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