Hedgehog pathway blockade with the cancer drug LDE225 disrupts taste organs and taste sensation

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While this paper was under revision Yang et al reported effects on taste bud cells in the circumvallate papilla in mice treated with Vismodegib for 15 weeks (Yang, Cong JS Yoon, JM Egan. 2014. Cancer Medicine Open Access, doi:10.1002)

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Taste sensation on the anterior tongue requires chorda tympani nerve function and connections with continuously renewing taste receptor cells. However, it is unclear which signaling pathways regulate the receptor cells to maintain chorda tympani sensation. Hedgehog (HH) signaling controls cell proliferation and differentiation in numerous tissues and is active in taste papillae and taste buds. In contrast, uncontrolled HH signaling drives tumorigenesis, including the common skin cancer, basal cell carcinoma (BCC). Systemic HH pathway inhibitors (HPIs) lead to BCC regression but these drugs cause severe taste disturbances. We tested the hypothesis that taste disruption by HPIs reflects a direct requirement for HH signaling in maintaining taste organs and gustatory sensation. In mice treated with the HPI LDE225 up to 28 days, HH-responding cells were lost in fungiform papilla epithelium and papillae acquired a conical apex. Taste buds were either absent or severely reduced in size in more than 90% of aberrant papillae. Taste bud remnants expressed the taste cell marker Keratin 8 and papillae retained expression of nerve markers, neurofilament and P2X3. Chorda tympani nerve responses to taste stimuli were markedly reduced or absent in LDE225-treated mice. Responses to touch were retained, however, whereas cold responses were retained after 16 days of treatment but lost after 28 days. These data identify a critical, modality-specific requirement for HH signaling in maintaining taste papillae, taste buds and neurophysiological taste function, supporting the proposition that taste disturbances in HPI-treated patients are an on-target response to HH pathway blockade in taste organs.
INTRODUCTION

Taste sensation on the anterior tongue is relayed via chorda tympani nerve fibers that connect and synapse with receptor cells in taste buds in fungiform papillae and anterior folds of the foliate papillae (Breslin and Spector 2008; Krimm and Hill 2000; Mistretta 1991). Taste buds include 50-100 differentiated cells spanning the depth of the lingual stratified squamous epithelium (Chaudhari and Roper 2010). The cells terminate apically in microvilli that are exposed to the oral environment and incorporate receptor proteins and ion channels to initiate taste transduction.

Taste cells differentiate from surrounding epithelium (Liu et al. 2013; Okubo et al. 2009) and turn over in a continuous replacement cycle with life spans from 3 to 30 days (Hamamichi et al. 2006; Perea-Martinez et al. 2013); thus, they are susceptible to environmental, metabolic and pharmacologic agents that affect the cell cycle. However the regulation of taste cell differentiation and turnover and maintenance of sensation via the chorda tympani nerve are not well understood. A principal regulator of cell and tissue homeostasis is the Hedgehog (HH) signaling pathway (Barakat et al. 2010). In the taste system Sonic hedgehog (SHH) is localized within taste buds, positioned for paracrine signaling to HH-responding cells in perigemmal, basal epithelial and stromal cells (Liu et al. 2013). We suggest that HH signaling maintains neurophysiological taste function, a proposal that has not been examined.

In contrast to maintaining tissues, uncontrolled HH signaling drives development of basal cell carcinoma (BCC), a common skin cancer (Teglund and Toftgard 2010). Patients with BCC are treated with HH Pathway Inhibitors (HPIs) that block Smoothened, a core HH pathway component (Lin and Matsui 2012). Although HPIs lead to BCC regression, patients taking these drugs report severe taste disturbance (Sekulic et al. 2012). After using the HPI vismodegib for two months, 80% of patients complain of taste disruption and often discontinue treatment due to taste effects (Tang et al. 2012). We suggest that the taste disturbance associated with HPI use relates to taste bud and chorda tympani nerve disturbance when HH signaling is inhibited.

We treated mice with Vehicle or the HPI-drug LDE225 (erismodegib, sonidegib) by oral gavage for 16 or 28 days, and examined gustatory tissue and electrophysiological chorda
We show that pharmacologic HH pathway blockade disrupts taste organs and chorda tympani responses to all taste qualities, resulting in severe defects in taste sensation. Intriguingly, the functional effects are modality specific, with tactile and temperature sensation retained.

MATERIALS and METHODS

Animals. Adult female, Gli1lacZ/+ reporter mice (Bai et al. 2002; Jackson Laboratory Strain 003081), used to monitor the expression of Hedgehog signaling, or C57BL/6 mice (Charles River) were used, under National Institutes of Health and University of Michigan Animal Care and Use Committee approved protocols.

Experiments. Mice were treated by daily oral gavage for two weeks (14-16 days; n = 10 LDE225−, 7 Vehicle-treated) or 4 weeks (28-29 days; 6 LDE225−, 6 Vehicle-treated) with LDE225 (ChemieTek NVP-LDE225) dissolved in Vehicle (PEG 400/5% dextrose in water) at a dose of 20 mg/kg, or Vehicle alone. The dose was determined from data in Pan et al (2010) and in preliminary studies. The oral gavage probe (22ga, 25mm; Instech, PA) by-passed all oral tissues, for direct delivery into the stomach. Treatments were 14-16 days to encompass the ‘average’ 10 day life span of taste bud cells (Beidler and Smallman 1965), and 28-29 days to encompass about three ‘average’ life spans. We refer to treatment times as 16 or 28 days.

After gavage, tongues were collected and prepared for tissue analysis, or electrophysiological responses were recorded from the chorda tympani nerve and then tongues were collected.

Tissue preparation. Tongues were fixed overnight at 4°C in 4% PFA in 0.1M PBS, cut anterior to the intermolar eminence, bisected, and one half transferred to 70% ethanol, for paraffin embedding and serial sagittal sections at 6 µm. The other half tongue was cryoprotected with 30% sucrose in PBS and frozen in O.C.T. Serial sagittal sections were cut at 10 µm and mounted for immunostaining (Liu et al. 2013).
Primary antibodies were: Goat anti-SHH (AF464, 0.1 µg/ml, R&D Systems); Rat anti-Keratin 8 (TROMA-1, 1:1000, Developmental Studies Hybridoma Bank); Rabbit anti-Neurofilament- H (NB300-135, 1:1000, Novus Biologicals); Rabbit anti-P2X3 (NB100-1654, 1:2000, Novus Biologicals). Secondary antibodies were Alexa fluor 488 or 568 (Invitrogen, 1:500). For X-Gal staining, frozen sections from Gli1lacZ/+ reporter mice (16 day treatment) were dried, rehydrated and transferred into X-Gal solution (Liu et al. 2013). Procedures for scanning electron microscopy (SEM) were as described (Liu et al. 2013).

Electrophysiology. Mice were anesthetized with ketamine/xylazine mixture (80-100 mg/kg, ketamine; 5-10 mg/kg, xylazine, i.p.) and maintained with ketamine (80 -100 mg/kg). The chorda tympani was dissected, cut centrally and placed on a recording electrode, with a reference electrode nearby. Amplified neural activity was connected to an A/D converter (Cambridge Electronic Design) and passed through an integrator circuit (Shimatani et al. 2002). Tactile and thermal stimuli consisted of stroking the anterior tongue quadrant 5-7 times over about 5 sec with a glass rod or applying 4ºC water. Chemical stimuli were dissolved in room temperature, distilled water and 30 ml applied on the tongue by gravity flow at 3 ml/sec: 0.1 and 0.5M NaCl; 0.1 and 0.5M NH₄Cl; 0.01N HCl; 0.03 and 0.1M citric acid; 1.0M sucrose; 0.1M MSG; 0.02M quinine HCl. Chemicals remained on the tongue for 20 s, followed by a distilled water rinse for at least 30 s. To monitor stability, NaCl and NH₄Cl were applied 3-4 times and the entire stimulus series was repeated.

Data Analysis

Papilla and taste bud quantification. On each half tongue, 600 µm of the mid-region of tissue was analyzed, to exclude sections with the median furrow or the lateral edges. In serial sections, fungiform papillae and resident taste buds were categorized and counted as:

1. Typical Fungiform Papilla and Typical Taste Bud: The rectangular papilla has a broad connective tissue core covered by a thin epithelium with a single apical taste bud. Cells of the bud span the depth of the epithelium and form an apical taste pore. The papilla core includes
stromal cells and nerve fibers. 2. **Atypical Fungiform Papilla and Atypical Taste Bud**: The papilla is misshapen with multiple, cornified apical layers that form a pointed cap. Taste buds have reduced cells and lack a taste pore that traverses the cornified layers. 3. **Atypical Fungiform Papilla and No Taste Bud**: The papilla is as described in Category 2 and there are no discernible taste cell collections.

**Electrophysiology.** Because response magnitude represents activity from a large number of compound action potentials, data are quantified by summation. For each stimulus, the height of the steady-state portion of the summated recording above baseline, at 5 s after stimulus application, was measured as response units in mm. To compare chorda tympani responses, the magnitudes can be normalized or expressed as ratios relative to a standard stimulus, such as 0.5M NH₄Cl. However, analyzing the current data in this way distorts the dramatic effects because LDE225 treatment can effectively eliminate responses to all chemical stimuli. Therefore we used absolute measures of taste-evoked activity and plotted mean values for taste responses. Amplifier settings were unchanged across recordings.

**Statistics.** We used two-way ANOVA to compare differences for each papilla morphology category, with main effects of treatment or duration of treatment, and group-wise comparisons with the Least Significant Difference (LSD) approach for LDE225 versus Vehicle treatment at each duration, and for 16 versus 28 day duration for each treatment type. The independent samples t test, with Levene’s test for equality of variance, was used to compare the magnitude of chorda tympani responses for each chemical between treatments. For responses to cold stimuli, we used two way ANOVA to assess main effects for treatment and duration, and LSD for within treatment, or duration, comparisons. Data in figures are presented as Mean ± S.E.M. Significance was set at \( p < 0.05 \) but all significance levels are stated within the text.
RESULTS

**HH pathway inhibition alters papilla and taste bud morphology and numbers.** LDE225 treatment profoundly alters fungiform papilla and taste bud morphology (Fig. 1A). Compared to Vehicle-treatment there is a loss in papilla integrity to acquire a conical tip and loss of taste buds to a few cell remnants.

In LDE225-treated mouse tongues only 0-7% of papillae are Typical compared to >90% in Vehicle-treated mice (Fig. 1A, Typical Fungiform Papilla (FP) and Typical Taste Bud (TB); F(1,25) = 2562.2, p<0.001; 16 day Vehicle versus LDE225, p<0.001, 28 day Vehicle versus LDE225, p<0.001). Furthermore, after LDE225 gavage, 48-66% of the fungiform papillae have acquired a conical apex with thick layers of keratin compared to 2-3% with Vehicle-treatment (Fig. 1A, Atypical FP and Atypical TB; F(1,25) = 192.1, p<0.001); 16 days between Treatment, p<0.001; 28 days, p<0.001). Some of these atypical, hyperkeratinized papillae contain disoriented taste bud cells with or without a taste pore, but no taste buds are Typical. Further, in LDE225-treated mice 27-52% of fungiform papillae had no taste buds compared to only 2-7% in Vehicle-treated (Fig. 1A, Atypical FP and No TB; F(1,25) = 92.2, p<0.001; 16 days between treatment, p<0.001; 28 days, p<0.001).

In addition, within each Papilla and Taste Bud Category (Fig. 1A) there were significant overall differences between 16 and 28 day durations (each Category: F(1,25) values = 9.9, p=0.004; F=5.1, p=0.033; F(1,25) = 15.7, p=0.001). With posthoc tests, it was apparent that the differences were for duration between LDE225-teated groups, shifting to more extreme acquisition of Atypical papillae without taste buds (p values=0.01 to < 0.001).

Overall, compared to Vehicle-treatment, mice after LDE225 gavage have a remarkable decrease in typical papillae and taste buds. Furthermore, HPI treatment extended to 28 compared to 16 days results in a significant increase in the Atypical FP and No TB category. To confirm that HPI treatment reduced HH signaling in fungiform papillae, we analyzed tongues of Gli1lacZ/+ reporter mice after 16 days treatment. Compared to Vehicle, Gli1
expressing, HH-responding cells (X-Gal positive) were absent in the basal layer of the papilla epithelium and in perigemmal cells in LDE225-treated tongues, but were detected in the papilla stroma cells (Fig. 1B). Therefore, LDE225 effectively blocks HH pathway activation in the papilla epithelium, where putative taste bud progenitors reside.

Filiform papillae after HH inhibition. In contrast to profound effects on fungiform papillae and taste buds, nongustatory, filiform papillae are not altered with HPI treatment. After 16 and 28 days, the stereotypic orientation of filiform papillae was intact in LDE225- compared to Vehicle-treated tongues (Fig 1C). SEM also confirmed the atypical fungiform papillae with spinous cap and absence of taste pore in LDE225-treated tongues (Fig. 1C insets).

Residual taste bud cell remnants and papilla innervation after HPI treatment. In taste buds from LDE225-treated tongues, SHH was much reduced, as expected in association with a reduction in taste bud size (Fig. 2A 16 days; 2B 28 days, K8/SHH). Remnants of taste bud cells that remained after HPI treatment were labeled with the taste bud cell marker Keratin 8 (K8), and taste bud cells were markedly reduced in number (Fig. 2A,B K8/SHH insets). SHH was detected in a subset of the K8-positive, taste bud cells. Because only 0-7% of papillae and taste buds were Typical after 16 or 28 days of HPI treatment (Fig 1A), there were in fact extremely few intact taste buds on the tongue.

Sustained innervation was observed in papillae of LDE225-treated tongues, comparable to that detected in Vehicle-treated tongues after 16 or 28 days treatment (Fig. 2A,B K8/NF). However, an apical ‘basket’ of NF-positive innervation seen in Vehicle-treated tongue is not obvious with LDE225 treatment, in association with taste bud cell reduction or loss.

Taste nerves express P2X3 receptors that are essential for synaptic transmission between afferent innervation and taste cells (Finger et al. 2005; Ishida et al. 2009). P2X3, therefore, marks afferent taste fibers. Notably, taste fibers were retained in the papilla core of LDE225- compared to Vehicle-treated mice (Fig 2A,B K8/P2X3). Overall, results suggest that the effect of the HPI LDE225 is principally epithelial and does not directly impair papilla and
gustatory innervation. However, further study can clarify the precise effect of HH pathway inhibition on the extent of innervation.

**Reduced taste nerve responses to chemical stimuli after HH pathway inhibition.** Chorda tympani recordings after 16 days gavage in Vehicle and LDE255-treated animals illustrate nerve responses (Fig. 3A). Responses were obtained to all taste stimuli in mice with Vehicle treatment. Salt and acid responses were of higher magnitude than responses to sweet, bitter or umami stimuli.

Notably, responses to chemical stimuli were reduced substantially or eliminated after 16 day HPI treatment. Integrated responses to the chemical stimulus sequence from two LDE225-gavaged mice (Fig. 3A) illustrate that although the response to each chemical was profoundly reduced in each mouse, the degree of response reduction differs. Overall, residual responses comparable to those in Fig. 3A were recorded in 3 animals after HPI gavage (LDE Residual) whereas there were no responses from the other 3 animals (LDE225 No response). Residual responses typically were apparent for the higher concentrations of NaCl, NH₄Cl and citric acid. Stimulation with the other taste qualities, and HCl, was ineffective.

Average responses from 16 day LDE225-treated mice (Fig. 3B) demonstrate significantly reduced responses for all chemicals (t values = 3.2 to 5.9, p = 0.01 to 0.03), except sucrose and MSG where the p values were marginal (p = 0.07, 0.11).

After 28 days of LDE225 treatment, responses to taste stimuli were effectively eliminated (Fig 3A). All comparisons of LDE225- with Vehicle-treated chemical responses were highly significant (Fig. 3B; 28 days, t = 3.7 – 11.9; p = 0.0001 – 0.02), except quinine HCl (p = 0.18).

Thus profound reductions or complete loss of chemical responses were observed after 16 days of HPI and after 28 days taste responses were absent.

**HPI treatment and responses to touch and cold.** The chorda tympani responds to lingual touch and cold stimuli (Ogawa et al. 1968; Finger et al. 2005). Because responses to touch stimuli are rapidly adapting, and not sustained, summated responses are not a valid
representation of the neural data. Therefore we present ‘raw’ recordings for tactile stimulation (Fig 4A).

Although 16 days of LDE225 treatment reduced or eliminated responses to chemicals, responses to touch and cold stimuli in the chorda tympani receptive field were not affected (Fig. 4A,B). After 28 days of LDE225 treatment tactile responses still were obtained from all mice (Fig. 4A). However, cold responses were lost (Fig. 4B).

There was an overall difference in treatment effect for cold responses between 16 and 28 day duration (F(1,14) = 4.99, p=0.04). Posthoc analysis indicated that the difference was at the 28 day (p=0.004), not 16 day, duration. Further, for LDE225 treated mice, the difference in cold responses between 16 and 28 durations was profound (F(1,14)=23.2, p<0.001).

The data indicate that alterations in chemical response magnitude did not result from a generally disrupted innervation and that effects of HH inhibition were modality specific. Further, with longer LDE225 treatment, chemical responses were no longer obtained in any HPI-treated mice and cold responses were lost.

**DISCUSSION**

This study is the first to demonstrate that HH signaling is a principal regulator of papilla and taste bud maintenance and neurophysiological taste sensation. We show that the HPI LDE225 profoundly affects the peripheral taste system, including altered fungiform papilla morphology, reduced taste bud numbers, and extreme reductions in or loss of chorda tympani nerve responses to all taste qualities.

BCC patients treated with HPIs experience side effects including “taste disturbances” of decreased taste perception, loss of taste or abnormal taste (Tang et al. 2012; Sekulic et al. 2012). Observed changes in taste sensation in LDE225-treated mice can explain the alterations in taste perception in patients treated with HPIs. Although defects in taste are common in treatment with various anti-neoplastic agents, our data suggest that the taste effects with HPI
treatment reflect a specific requirement for physiologic HH signaling in adult taste organ
structure and function that is disrupted in HPI-treated patients.

**Taste papilla and taste bud effects and innervation.** SHH ligand is produced by taste bud
cells for signaling in a paracrine manner to HH-responsive cells in the basal epithelial layer,
perigemmal cells, and stromal cells of the papilla (Liu et al. 2013). With HPI treatment we now
demonstrate loss of HH pathway activity (Gli1lacZ/+ expression) in HH-responding cells, primarily
in the papilla epithelium. We propose that blockade of epithelial HH signaling leads to the
profound alterations in taste bud structure and function.

After LDE225 treatment fungiform papillae exhibit a strikingly atypical morphology. The
apical papilla cells acquire a spinous keratin cap reminiscent of filiform papillae, supporting
the specific proposition that HH signaling is required to maintain the fungiform papilla (Liu et al.
2013). Further, the K8+ taste bud remnants situated under this spinous cap lack a taste pore.
Although the papilla structure is not intact, NF+ and P2X3+ nerve fibers remain within the
papilla core, indicating that HPI treatment affects taste organs themselves and does not grossly
disrupt the innervation. Furthermore, nongustatory, filiform papillae are not obviously altered.

**Chorda tympani responses to taste stimuli.** In LDE225-gavaged mice chorda tympani nerve
taste responses were reduced or eliminated after 16 days, and eliminated after 28 days.
Logically, response reductions could relate to reduction in taste buds. The residual taste
responses that we observed in half of the 16 day, LDE225-treated animals might be attributed to
retention of some ‘typical’ papillae and taste buds in treated tongues. Further, the residual,
measurable responses to NH4Cl and citric acid, in particular, could suggest that some taste cells
are more gradually susceptible to HPI treatment, possibly related to variable turnover cycles
of taste bud cells and response properties of specific cell types (Perea-Martinez et al. 2013).

**Chorda tympani responses to touch and temperature stimuli.** In contrast to taste,
responses to lingual touch and cold in the chorda tympani were not affected after 16 days
LDE225 treatment, and touch responses remained after 28 days. Presumably the tactile and
thermal fibers terminate in either mechanoreceptors or thermal receptor endings, not transduced via taste bud cell receptors. Notably, chorda tympani response differences across modalities also were reported in knockout mice lacking P2X3 receptors, required in synaptic transmission between taste afferent fibers and taste bud cells (Finger et al. 2005). In these mice, although all taste responses were eliminated, responses to touch and temperature remained.

Our results suggest that mechanical and thermal modalities could be ‘spared’ the effects HH pathway inhibition in the taste system. However, this is complicated by reports that the chorda tympani innervates taste buds only, not surrounding papilla epithelial cells (Whitehead et al. 1985); thus, tactile and thermal nerve endings presumably would be within the taste bud. It is possible that responses to touch and thermal stimuli originate from receptors adjacent to the taste bud, suggesting a broader distribution of the chorda tympani receptive field rather than only within the taste bud. Overall, the data clearly indicate that HPI treatment differentially affects oral sensations and demonstrate the importance of updated, accurate maps of taste, touch and thermal receptors in fungiform papillae.

In summary, our study establishes: an essential and modality-specific requirement for HH signaling in maintaining neurophysiological taste sensation in mice, underlying the likely cause of taste disruption in HPI-treated patients. We propose that taste disturbances in HPI-treated patients derive from HH-dependent loss of taste papilla integrity and taste buds, with a specific, concomitant reduction of peripheral nerve taste responses that transmit taste sensation centrally. Our results predict that although taste disruption in HPI-treated patients will be profound, other lingual modalities of touch and temperature may be spared.
REFERENCES


FIGURE LEGENDS

**Figure 1.** Fungiform papillae and taste buds are altered after HPI treatment for 16 or 28 days.

A. Hematoxylin and eosin sections demonstrate Fungiform Papilla (FP) and Taste Bud (TB)
Categories: Typical FP and Typical TB; Atypical FP and Atypical TB; Atypical FP and No TB.

Histograms present percentage of FP and TB Types in Vehicle- or LDE225-treated mice. After LDE225 treatment, only a very small percentage of Typical FP and TB remain. Asterisks denote significant differences between treatments and between 16 and 28 day treatment periods and illustrate even more Atypical FP and No TB at 28 compared to 16 days treatment (*p<0.001; **p<0.01).

B. In Gli1lacZ/+ reporter mice, HH-responding, ß-Gal positive, cells in the papilla epithelium are eliminated after 16 days LDE225 gavage. Stromal ß-Gal positive cells remain. Dashed lines demarcate the epithelial border and a circle denotes the taste bud, from surrounding perigemmal cells.

C. Scanning electron micrographs (SEM) for Vehicle- and LDE225-treated tongues illustrate intact filiform papillae and orientation. Fungiform papillae are indicated with a circle. Insets represent misshapen fungiform papillae with a cornified cap (asterisk) and no taste pore after LDE225-treatment. The fungiform papilla in the Vehicle SEM has a taste pore (arrow). Scale bars apply to all images in respective panel.

**Figure 2.** SHH expression and taste bud cells are reduced, but innervation remains after HPI treatment for A, 16 days or B, 28 days. K8/SHH: SHH expression remains in taste bud cell remnants; but K8+, taste cells are much reduced, see insets. Dashed lines demarcate the epithelial border. K8/NF and K8/P2X3: With NF and P2X3 (taste fiber specific) immunoreactions, innervation is detected within the papilla core in Vehicle- and LDE225-treated tongues (arrows indicate P2X3+ fibers). Scale bar applies to all images. The asterisk in K8/SHH and K8/NF panels indicates nonspecific, surface staining.

**Figure 3.** Chorda tympani responses to taste stimuli are reduced or eliminated with LDE225 treatment. A. Top three traces illustrate summated responses to salt, acid, sweet, bitter and umami stimuli in a Vehicle-treated and two LDE225-treated mice, after 16 days. Residual responses to salts and citric acid, illustrated in the middle trace, were retained in half of HPI-treated animals. Blue boxes highlight responses across recordings. The fourth trace presents chorda tympani recordings after 28 days LDE225 treatment. B. Average chorda tympani responses (means, standard errors) for Vehicle- or LDE225-treated mice, after 16 or 28 days, were significantly different. Some response to salt or citric acid stimuli was retained after 16 days of LDE225 treatment in 3 mice, while in 3 mice all chorda tympani responses to chemicals
were eliminated. After 28 days of LDE225 treatment essentially no chemical responses were observed. *p<0.05.

**Figure 4.** Chorda tympani responses to tactile and cold stimuli are retained after LDE225 treatment. Recordings are presented of raw tactile responses (A, Tactile) or integrated responses and histograms for cold stimulation (B, Cold). Note that while responses to tactile stimuli are retained throughout LDE225 treatment, cold responses are absent after 28 days of HPI treatment. Numbers of mice are noted within bars. *p<0.001; **p<0.01.
FIGURE 1
FIGURE 2
FIGURE 3
FIGURE 4