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RESEARCH ARTICLE



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In vitro effects of sodium butyrate, a histone deacetylase inhibitor on canine mammary tumour subtypes

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ABSTRACT

Malignant canine mammary tumours (CMTs) are subdivided as carcinomas (C), sarcomas (S) and carcinosarcomas (CS) according to their histological type. Due to the varying responses to different treatments, novel therapeutic approaches are required. Sodium butyrate (NaBu) is the first histone deacetylase inhibitor and exhibits anti-cancer activity in different types of human cancers. However, no study is available in the literature evaluating the effects of NaBu on CMT cells. Therefore, for the first time, we aimed to investigate the *in vitro* efficacy of NaBu on three subtypes of CMT cells. Primary cells were isolated from neoplastic tissues of three dogs with CMT. Cell viability, apoptotic percentage and changes in the cell morphology, apoptosis-associated gene and protein expression levels were evaluated. Our findings showed that NaBu decreased the growth of the cells and increased the percentage of apoptosis in three subtypes of CMT cells. Additionally, NaBu treatment affected the expression levels of genes and proteins associated with apoptosis, especially in S cells. Our preliminary results, for the first time, revealed the efficacy of NaBu in CMT cells and the importance of the histological subtype in NaBu response. However, the molecular classification of CMT and the underlying mechanisms of the different NaBu responses should be further investigated.

KEYWORDS

apoptosis, canine mammary gland tumour, histone deacetylase inhibitor, sodium butyrate

INTRODUCTION

Mammary tumours are the most common neoplasms in bitches, like their human counterparts, with the highest incidence of 25–42% among companion animals (Klopffleisch et al., 2011). Canine mammary tumours (CMTs) can serve as a study model for human breast cancers due to their similar clinical course and molecular mechanisms. Shared clinical features are spontaneous occurrence, age of onset, hormonal aetiology, clinical staging, lymph node invasion and most common histological type. At the molecular level, some parameters, including expression of steroid hormones and epidermal growth factor, the effectiveness of biomarkers as a proliferation index, the occurrence of *p53* mutation, and overexpression of metalloproteinase and cyclooxygenase can also be investigated in dogs (Queiroga et al., 2011). The surgical approach is currently the most commonly used treatment for CMTs. However, it is considered an extremely invasive method with certain complications such as posterior limb oedema, hematoma, dehiscence and contamination of the surgical site; subcutaneous

emphysema, infection, bleeding, seroma, hyperesthesia, allodynia, pain, changes in the patient's blood profile due to long-term anaesthesia, exhausting post-operative care, and thus, distress in the patient (Aiello, 1998; Al-Asadi et al., 2010). Because of these complications and unsatisfactory outcomes of the other adjuvant therapies, new targeted treatment options for CMTs are still needed.

Malignant CMTs can be subdivided according to their cellular origin. Although most malignant CMTs are of epithelial origin (carcinomas; C), some are mesenchymal neoplasms (sarcomas; S). Malignant CMTs may contain both epithelial and myoepithelial components, as well as areas of cartilage and bone tissues (mixed carcinosarcomas; CS). While complete surgical excision is sufficient for simple carcinomas smaller than 3 cm, simultaneous chemotherapy is recommended for those larger than 3 cm. On the other hand, in CS, complete excision, radiotherapy and chemotherapy are recommended regardless of tumour size. Although wide-margin surgery is performed in S cases, the adjuvant treatment possibilities are still in an experimental phase and remain unsuitable for treatment due to the poor prognosis. In addition, many drugs used for chemotherapy cause acute or late treatment-related side effects (Sorenmo, 2003; Sorenmo et al., 2013; Lana and Dobson, 2016). Because of the heterogeneity and different responses, alternative therapeutic options that can inhibit cancer cell proliferation without general toxicity continue to be explored for all subtypes of CMTs.

Due to their reversibility, epigenetic changes have recently been proposed as new treatment strategies. The epigenetic changes depend on the functioning of special enzymes such as DNA methyltransferase and histone deacetylase (HDAC), which are the first targets for epigenetic therapy (Esteller, 2008; Handel et al., 2010). Therefore, an epigenetic therapy based on inhibiting these enzymes can reveal anti-tumorigenic effects (Egger et al., 2010).

HDAC inhibitors (HDACi) induce the accumulation of acetylated histones by inhibiting histone deacetylase enzymes and affecting cellular processes in cancer cells (Fang and Ji, 2009). HDACi can be divided into four groups: short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides and benzamides. There are different forms of HDACi for the treatment of both haematological and solid tumours. Additionally, combining isoform-selective HDACi with anti-cancer agents will be a promising alternative for treating breast cancer (Namdar et al., 2010).

Sodium butyrate (NaBu) is the first HDAC inhibitor and inhibits cell growth via hyperacetylation of histone H3 and H4 in cancer cells (Candido et al., 1978). In the literature, the anti-cancer activity of NaBu has been evaluated in different types of human cancers, including colon, gastric and breast cancer cells (Cho et al., 2009; Wang et al., 2009, 2016; Shin et al., 2012; Salimi et al., 2017). However, there is no study in the literature evaluating the effects of NaBu on the subtypes of CMT cells. Therefore, we investigated, for the first time, the potential *in vitro* therapeutic efficacy of NaBu on three subtypes (C, CS and S) of CMT cells.

MATERIAL AND METHODS

Ethics approval

The dogs, included in this study, were operated for cancer treatment, not for experimental purposes. Tissue specimens removed during the surgery were used. The procedures conformed with the "Regulation on Working Procedures and Principles of Animal Experimental Ethics Committees Prepared by the Ministry of Environment and Forestry published in the Official Gazette dated February 2014 and numbered 28,914". Dog owners were informed and asked for written consent to allow the participation of their dog in the study.

Case description and tissue sampling

Mastectomy was performed in dogs referred to the clinic (Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Istanbul University-Cerrahpasa) with a palpable mass in their mammary glands preliminary diagnosed with mammary gland tumours. Mammary tissue samples, taken from a 2-year-old dog by total bilateral mastectomy and confirmed as healthy by histopathological examination, were used to evaluate the effect of NaBu on healthy canine mammary cells (HC). The tissue specimens, fixed in 10% neutral buffered formalin were submitted to the Department of Veterinary Pathology for histopathological evaluation. A portion of the unfixed fresh tissue samples was transferred to tissue culture (RPMI) medium for primary cell culture. For histopathological examination, the specimens were routinely processed, embedded in paraffin blocks, sectioned and stained with haematoxylin and eosin for light microscopic evaluation. Histopathological evaluation of the tumour samples was performed based on the criteria of Goldschmidt et al. (2011).

Case 1: A 10-year-old neutered female Golden Retriever weighing 33 kg was presented to the clinic with a palpable >5 cm mass recognized three months previously, and showing rapid growth in the last month. According to the owner, the patient's appetite and water intake were normal. On clinical examination, no abnormalities were detected in the regional lymph node palpation, heart rate, respiratory rate and rectal temperature. Abdominal ultrasonography, thoracic radiography, serum biochemistry and complete blood count did not reveal any significant abnormalities.

Case 2: An 11-year-old intact female Maltese terrier weighing 8 kg was presented to the clinic with multiple palpable masses (3 cm primary mass) that had been recognized a year ago. No abnormality was detected in clinical examinations except for unilateral lymph node hyperplasia.

Case 3: A 12-year-old female Chow-Chow dog weighing 26 kg was presented to the clinic with a five-month history of mammary mass (>5 cm). According to the owner, the bitch was neutered four months ago due to cystic endometrial hyperplasia-pyometra complex. No abnormality was detected during clinical examinations.



Primary cell culture and viability analysis

Primary cell isolation and culture protocol from CMTs and healthy tissues were conducted as previously described (Turna et al., 2021). We performed WST-1 cell viability assay to determine the cytotoxicity of NaBu on three subtypes of CMT and HC cells. For this purpose, an equal number of cells (2×10^4 cells/well) were seeded in 96-well plates and incubated for 24 h. The cells were then incubated with different (2.5, 5, 10 and 20 mM) concentrations of NaBu for 24 h. After this incubation with NaBu, WST-1 dye (Biovision, USA) was added, and the cells were incubated at 37 °C in a humidified incubator for 45 min. The absorbance values were determined using a microplate reader (Allsheng, China) at 450 nm. According to the cell viability results, we selected the most effective concentration of NaBu for further analysis.

Cell culture validation

The immunofluorescence (IF) method was performed to characterize the cultured cells and to visualize the expression of specific cell markers. Cells were cultured on a coverslip and fixed with 4% paraformaldehyde then blocked with goat serum. Subsequently, the cells were incubated with primary antibodies to CD44 (sc-18,849, Santa Cruz Biotechnology, CA, USA), to epidermal growth factor receptor (EGFR) (MA5-12875, Thermo, USA) and to estrogen receptor- α (ER- α) (ab137489, Abcam, Cambridge, UK) at room temperature for 3 h. Following this, the cells were incubated with secondary fluorescent antibodies Goat Anti-Mouse IgG H&L (AlexaFluor® 488) (ab150117, 1:1000, Abcam, Cambridge, UK) and Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (ab150081, 1:1000, Abcam, Cambridge, UK) at room temperature for 1 h. The nuclei of cells were stained with DAPI (4',6-diamidino-2-phenylindole) dye (Sigma, USA). Images were analysed with the EVOS Flouid Cell Imaging Station (Thermo Fisher Scientific, USA).

Annexin V analysis

Annexin V analysis was performed to evaluate the apoptotic effect of NaBu on HC and the three subtypes of CMT cells. An equal number of cells (1×10^5 cells/well) were seeded in 6-well plates. After a 24 h of culturing, 20 mM NaBu was added and the incubation continued for another 24 h. Then, the cells were detached with trypsin-EDTA (treatment and washed with PBS (Gibco, Thermo Fisher Scientific, USA). Afterwards, the cells were processed with the Muse Annexin V & Dead Cell Kit (Luminex Corporation, Austin, Texas, USA). After incubation for 30 min, the stained cells were analysed with the Muse Cell Analyzer (Lumiex), a miniaturized cytometry device for the quantitative differentiation of cells in different states (i.e., live, dead, early and late apoptotic).

Acridine orange staining

Acridine orange (AO) staining was performed to evaluate the morphological changes of the cells after NaBu treatment.

An equal number of cells (5×10^5 cell/well) were seeded and incubated on 6-well plates for 24 h, then 20 mM NaBu was added and the incubation was continued for an additional 24 h. Subsequently, the cells were fixed with 4% paraformaldehyde (Sigma, USA) for 30 min and washed with PBS. Staining was performed with $1 \mu\text{g mL}^{-1}$ AO (Sigma, USA) for 30 min. Finally, the cells were examined using an EVOS FLoid Cell Imaging Station (Thermo Fisher Scientific, USA).

Quantitative PCR and Western blot analysis

Quantitative PCR (qPCR) and Western blot analyses were conducted to determine the level of mRNA and the respective protein from the *bax*, *bcl-2* and *caspase-3* genes. An equal number of cells of the three CMT subtypes were grown in 6-well plates (1×10^6 cells/well). After 24 h of culture, the cells were treated with 20 mM NaBu for another 24 h, after which total RNA was extracted with the Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. The concentration of the extracted RNA was determined using a Qubit 4 Fluorometer (Thermo Fisher Scientific, USA). Then, total RNA was converted to cDNA with the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). The mRNA expression level of the *bax*, *bcl-2* and *caspase-3* genes was determined by the Step One Plus TM qPCR (Applied Biosystems) with TaqMan™ Gene Expression Assays Cf02727746_g1, Cf02741602_m1 and Cf02622236_m1, respectively. The *beta-actin* (Cf04931159_m1) was used as a reference gene.

For Western blot analysis, the total protein was extracted from the cells with RIPA lysis buffer (Santa Cruz Biotechnology, CA, USA). Concentration of the extracted protein was determined with a Qubit 4 Fluorometer (Thermo Fisher Scientific, USA). The protein fractions were separated by electrophoresis using Mini-PROTEAN TGX Stain-Free Protein Precast Gels in a Mini-PROTEAN Electrophoresis System (Bio-Rad, San Diego, CA, USA). Polypeptide bands were transferred to nitrocellulose membranes. The membranes were blocked in 5% nonfat milk in TRIS-buffered saline with 0.1% Tween 20, then treated with the primary antibodies to Bax (NB100-56096), Bcl-2 (NB100-56098) and Caspase-3 (NB600-1235) (Novus Biologicals, USA) overnight. Subsequently, the blots were reacted with horseradish-peroxidase conjugated secondary antibodies, namely Goat Anti-Mouse and Rabbit Anti-Goat IgG H&L HRP (ab6789 and ab6741, respectively) polyclonal antibodies (Abcam, Cambridge, UK) were added for 1 h. The protein bands were enhanced by the use of ECL Western Blotting Substrate Kit (Biovision, San Francisco, CA, USA) and recorded with a Syngene G:BOX F3 imaging system (Syngene, Cambridge, UK). For reference, the β -actin protein (sc-47,778; Santa Cruz Biotechnology, CA, USA) was used.

Statistical analysis

Statistical analysis was performed with the GraphPad Prism v8 software. The results were presented as mean $\pm$ standard



deviation of three independent experiments. One-way analysis of variance (ANOVA) followed by Tukey's test was performed for multiple comparisons. Obtained *P*-values less than 0.05 were considered statistically significant. A web-based software (<https://www.qiagen.com/tr/shop/genes-and-pathways/data-analysis-center-overview-page/other-real-time-pcrprobes-or-primers-data-analysis-center/>) was applied to evaluate the mRNA expression analysis results.

RESULTS

Histopathological findings

Case 1 was diagnosed as a solid (Grade 3) carcinoma (C). The tumour was arranged in a lobular pattern separated by a thin fibrovascular stroma. In some areas, the tumour cells of

varying shape from polygonal to oval, with moderate basophilic cytoplasm and unclear cell borders, were predominantly arranged in solid sheets and tubular structures. The nuclei were vesicular with one or two prominent basophilic nucleoli. Anisocytosis and anisokaryosis were moderate, and there were areas of necrosis. The mitotic count was 33 in 10 consecutive high-power fields (Fig. 1A).

Case 2, presented in Fig. 1B and C, was classified as a carcinosarcoma (CS). The tumour was composed of both epithelial and mesenchymal elements and exhibited infiltrative growth with a solid pattern. Epithelial neoplastic cells were arranged in a tubular fashion. The lining of the tubules was usually 1 to 2 cell-layer thick, with moderate anisocytosis. The mitotic figures were scarce. The mesenchymal component of the tumour consisted of neoplastic cells showing chondroid differentiation in some areas. Additionally, there were neoplastic areas of spindle cells within a

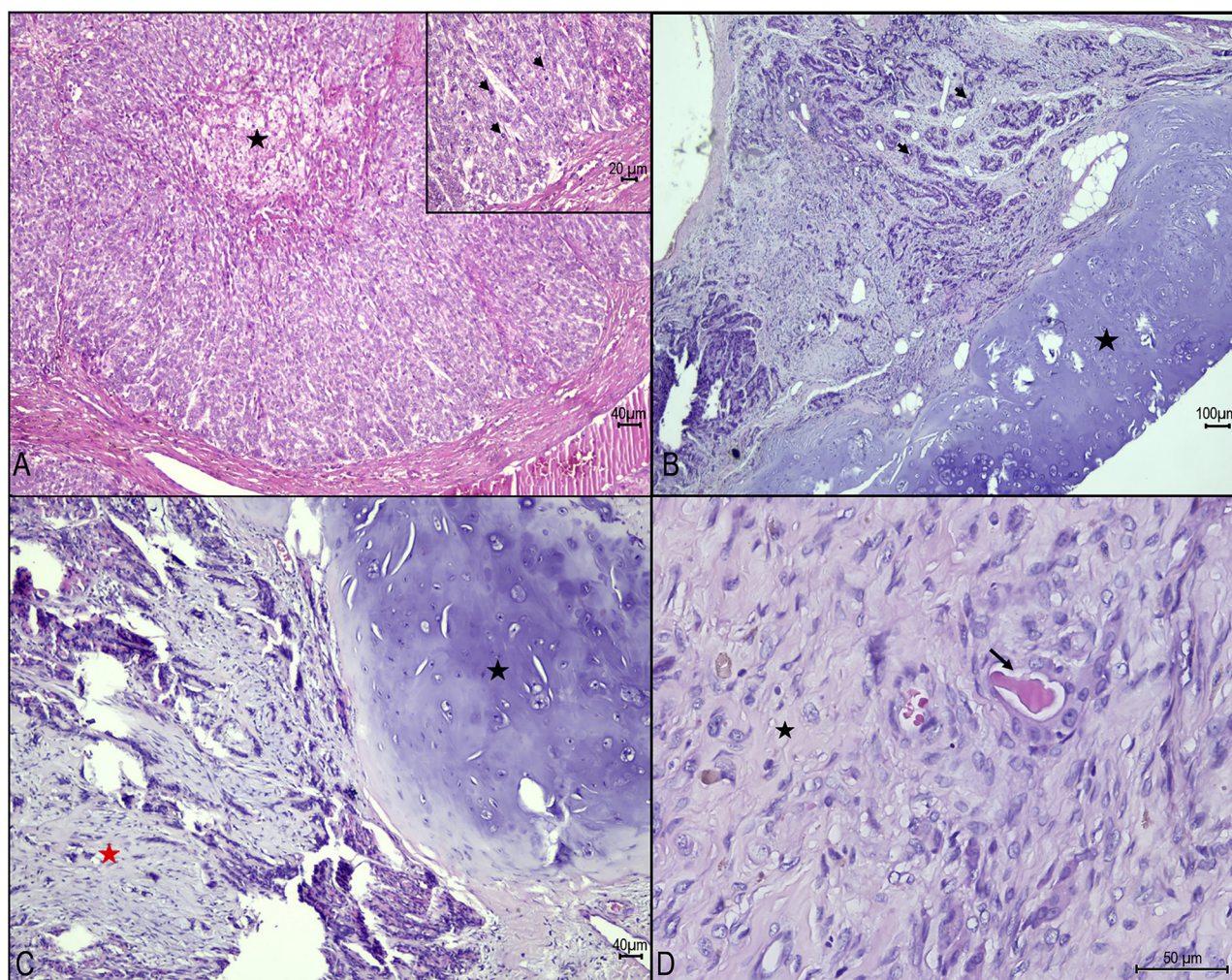


Fig. 1. Histopathological sections from the three types of canine mammary gland tumours (haematoxylin and eosin staining). Case 1 carcinoma. A: Anisocytosis, numerous mitotic cells (arrowheads) and a necrotic area (star) can be seen in a solid carcinoma of lobular pattern with thin fibrous stroma. Bar = 50 μ m. Case 2 carcinosarcoma. B: Tubule formation in the epithelial component of the tumour (arrows) and sharply demarcated sarcomatous region composed of malignant cartilage (star) tissue Bar = 100 μ m. C: Malignant cartilage (black star) and spindle cells among the irregularly shaped tubules (red star) within the myxoid matrix. Bar = 40 μ m. Case 3 sarcoma. D: Neoplastic cells with indistinct cell borders and elongated to oval nuclei within eosinophilic amorphous collagenous material (star), entrapped mammary tubule (arrow). Bar = 50 μ m

myxoid matrix. Intratumoral lymphocytic infiltration was also detected.

Case 3 was identified as a sarcoma (S). The tumour was composed of haphazardly arranged neoplastic cells with a background of dense, pink, amorphous collagenous material. The neoplastic cells had indistinct cell margins, with scanty eosinophilic cytoplasm, elongated and vesicular nuclei, and prominent nucleoli. Marked anisokaryosis and anisocytosis and wide areas of necrosis were also noted. The mitotic activity was low (Fig. 1D).

Histopathological examination of the healthy canine mammary tissue revealed low cellularity and high eosinophilia in the interlobular stroma, with no neoplastic lesions in the mammary glands and ducts.

Effect of sodium butyrate on cell viability

The effect of the different concentrations of NaBu on the HC and the three subtypes of CMT cells as evaluated by the WST-1 viability assay is summarized in Fig. 2. NaBu significantly decreased the viability of the S, C and CS cells in a dose-dependent manner without causing toxicity in HC cells. No significant cytotoxicity was observed after treatment with the 2.5 and 5 mM NaBu. After 24 h of 10 mM

NaBu treatment, the viability of S, C and CS cells was reduced to $79.5 \pm 1.0\%$, $80.0 \pm 1.5\%$ and $79.2 \pm 2.8\%$, respectively ($P < 0.01$). However, there was a significant reduction in the growth of S ($39.5 \pm 2.2\%$), C ($67.9 \pm 3.2\%$) and CS ($64.2 \pm 0.8\%$) cells upon 20 mM NaBu treatment ($P < 0.01$). Additionally, we observed less cytotoxicity ($85.3 \pm 0.5\%$) after 20 mM NaBu treatment in HC cells. Therefore, a 20 mM concentration of NaBu was selected for further experiments.

Effects of sodium butyrate on apoptosis

As shown in Fig. 3A, the results of the Annexin V assay demonstrated a significant ($P < 0.01$) increase in the percentage of both early and late apoptotic cells upon treatment with 20 mM NaBu in S, C and CS cells. The proportion of total apoptotic cells considerably increased to $39.3 \pm 0.4\%$, $32.4 \pm 0.6\%$, $32.3 \pm 0.9\%$ and $16.55 \pm 0.7\%$ in S, C, CS and HC cells, respectively ($P < 0.01$). We concluded that NaBu promoted apoptosis in the three subtypes of CMT. This was consistent with the WST-1 results. However, S cells were more sensitive to NaBu treatment than the other two (C and CS) tumour cells. At the same time, the 20 mM NaBu induced moderate apoptosis in the HC cells.

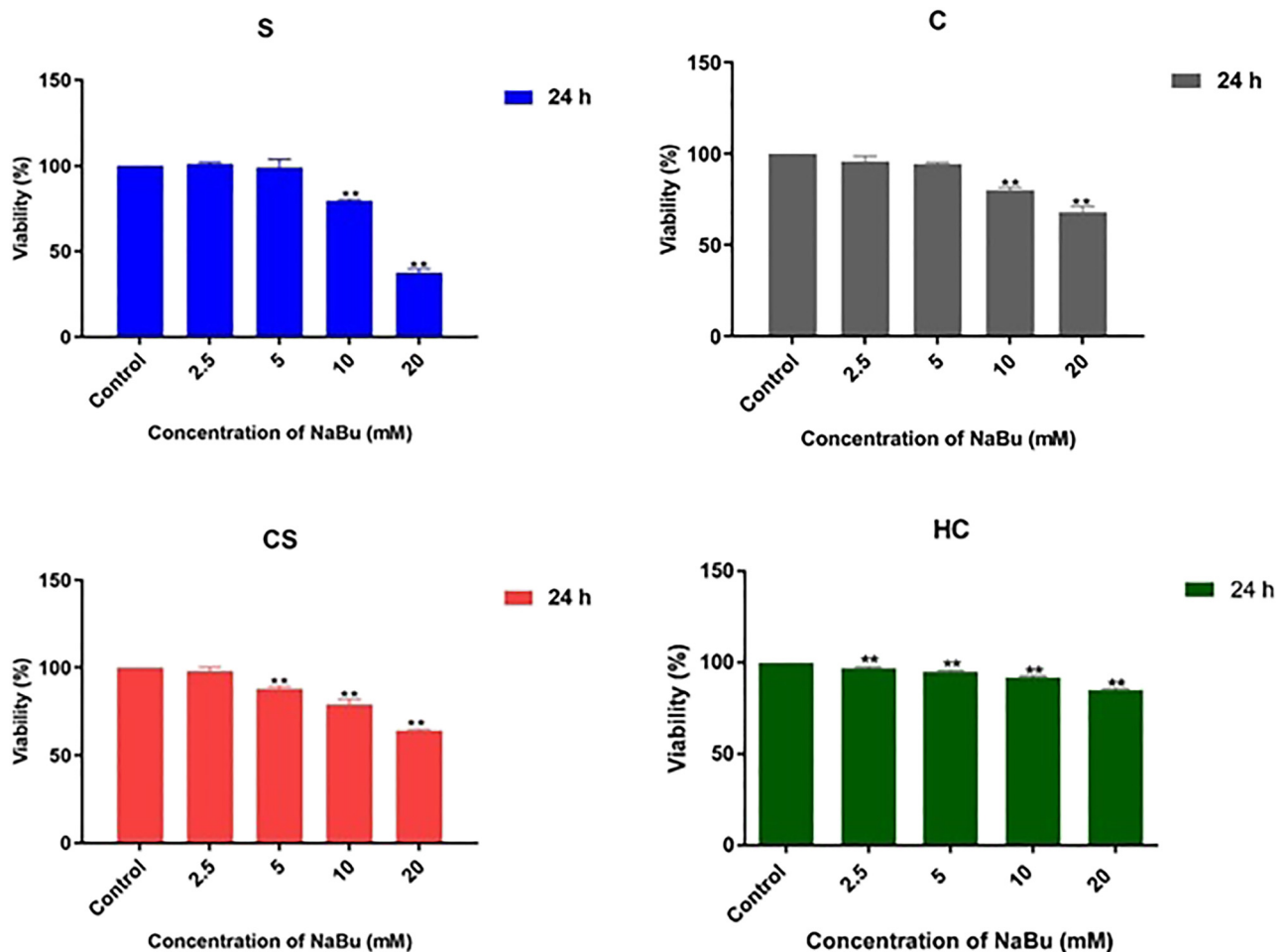


Fig. 2. Viability of canine mammary tumour (CMT) and healthy canine mammary (HC) cells after 24-h treatment with different concentrations of sodium butyrate (NaBu). *: $P < 0.05$; **: $P < 0.01$

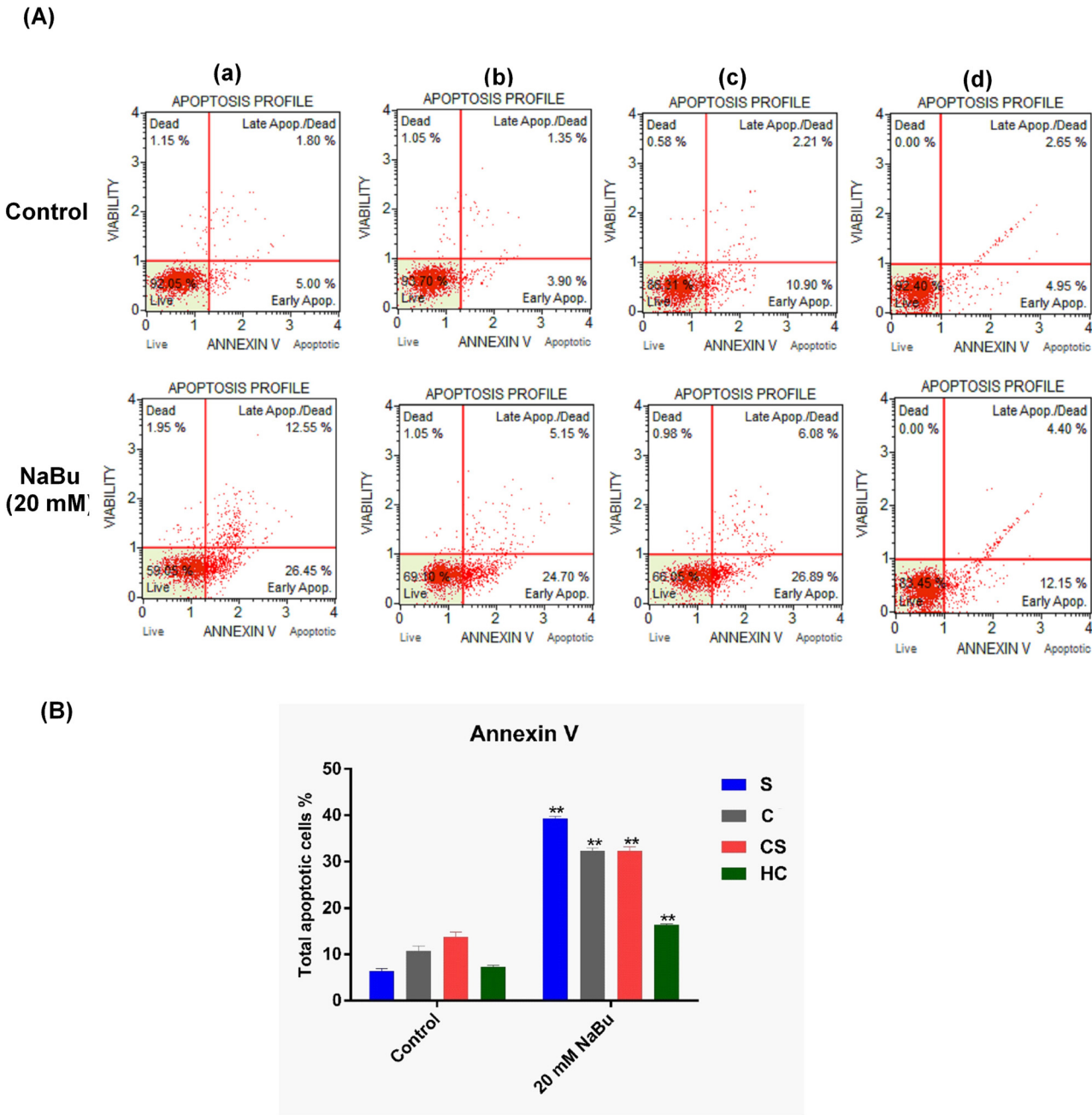


Fig. 3. Sodium-butyrate-mediated apoptosis in different CMT and HC cells. A: Histograms of the Annexin V assay after 20 mM sodium-butyrate (NaBu) treatment. B: Graphical representation of the total percentage of apoptotic cells in sarcoma (S), carcinoma (C) and carcinosarcoma (CS) tumours and in HC cells after treatment with 20 mM NaBu compared to the corresponding untreated controls. **: $P < 0.01$

Morphological changes of the cells after treatment with sodium butyrate

The NaBu-mediated apoptosis was further confirmed by the morphological changes of the S, C, CS and HC cells demonstrated with the AO staining as shown in Fig. 4. The untreated control group of each cell types underwent normal adherent growth. After NaBu treatment, however, the cells exhibited rounded shape. Ruptured plasma membranes and chromatin condensation could be observed particularly in S cells. Furthermore, the nuclei of the C and CS cells were irregularly shaped after the NaBu treatment. The most

striking changes were noted in the morphology of S cells, whereas no apoptotic morphological changes were detected in the treated HC cells.

Differences in the levels of apoptosis-related genes and proteins

The results of the qPCR and Western blot analyses are presented in Fig. 5. We found a 1.3, 2.4 and 2.3-fold increase, respectively, in the level of mRNA from the apoptosis-related *bax*, *bcl-2* and *caspase-3* genes in S cells after 20 mM NaBu treatment (Fig. 5A). On the other hand, lower

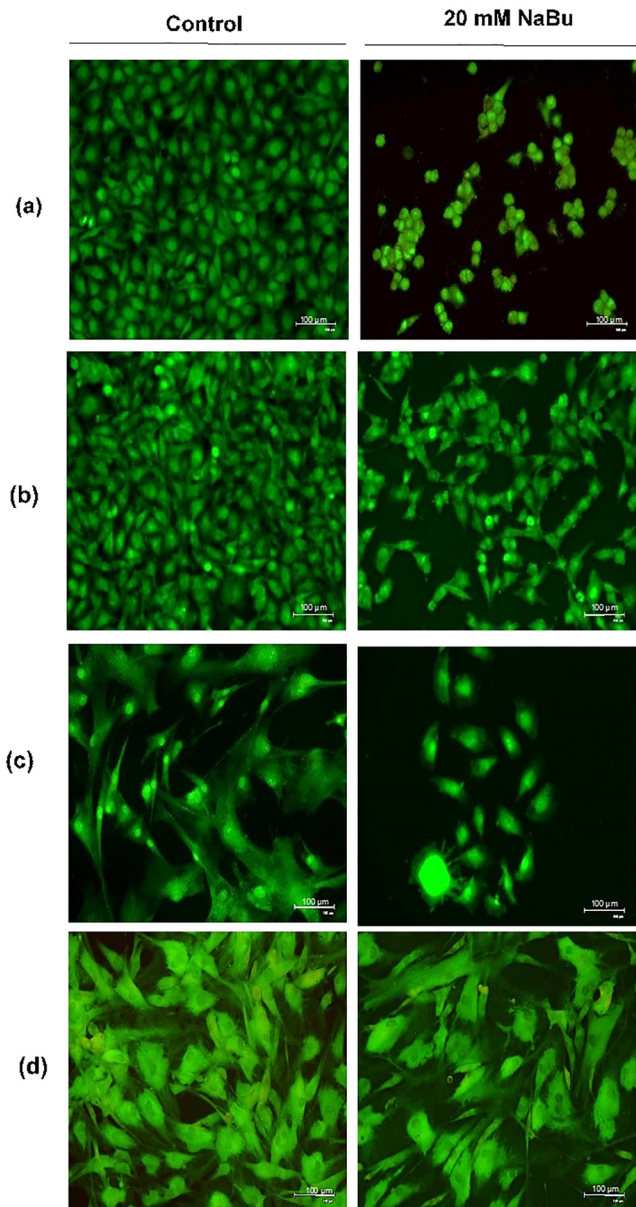


Fig. 4. Changes in cell morphology of different types of CMT and HC cells following treatment with 20 mM sodium-butyrate (NaBu) for 24 h. (a): sarcoma cells; (b): carcinoma cells; (c): carcinosarcoma cells; (d): healthy canine mammary gland cells (acridine orange staining)

expression levels of the *bcl-2* and *caspase-3* genes were detected in both C and CS cells treated with NaBu compared to the untreated control. Additionally, a 2.5-fold upregulation of the *bax* gene was detected in C cells (Fig. 5A). The results of the Western blot analysis, presented in Fig. 5B, largely supported the qPCR findings. Intriguingly, the 20 mM NaBu treatment did not increase significantly the level of the Bax and Caspase-3 proteins in S cells. Additionally, the increase in the Bax protein level in C cells did not seem proportional to the mRNA changes, especially if compared to the expected decrease in the Bax and Caspase-3 protein levels in CS cells (Fig. 5B).

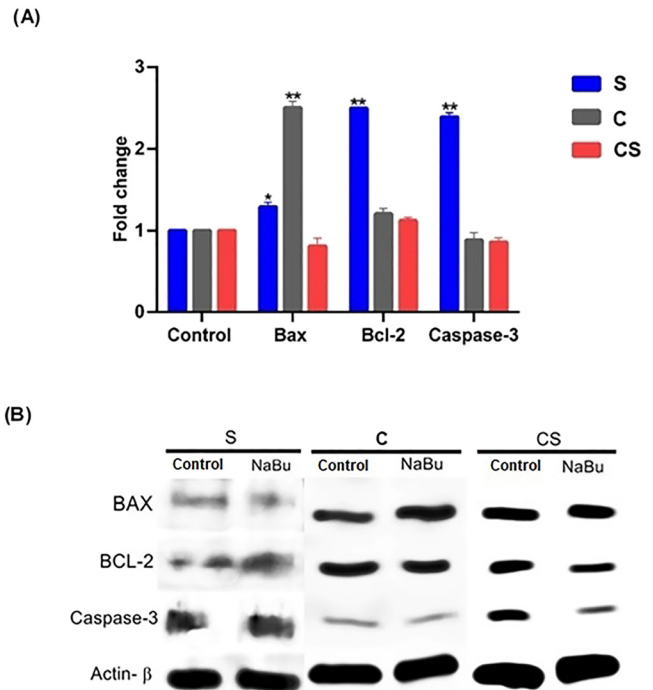


Fig. 5. Alterations in the expression levels of apoptosis-related proteins Bax, Bcl-2 and Caspase-3 in different types of CMT cells after treatment with 20 mM NaBu for 24 h. Control: untreated cells. C: carcinoma; CS: carcinosarcoma; S: sarcoma. A: mRNA levels of the genes as determined by qPCR. B: Western blot analysis of the proteins. Beta actin was used as protein control. *: $P < 0.05$; **: $P < 0.01$

DISCUSSION

For the first time in this study, we evaluated the role of epigenetic mechanisms inhibited by NaBu in three histological subtypes of CMT cells. Our findings showed that NaBu significantly inhibited the proliferation of CMT cells and induced apoptosis. The response of CMT cells to NaBu treatment differed depending on their histological subtype. Mainly, S cells were more sensitive to NaBu treatment than C and CS cells. Thus we provide novel data by showing the effect of NaBu on CMT cells and open a new perspective in revealing the possible role of HDACi in the treatment of CMTs.

HDACi has been reported to induce apoptosis and mitotic cell death via altering gene expression, cell cycle arrest and increasing ROS-induced cell senescence in different types of human cancers (Eckschlager et al., 2017; Autin et al., 2019). The drugs, called Vorinostat, Romidepsin, Belinostat and Panobinostat have been approved by the US Food and Drug Administration for cancer therapy (Autin et al., 2019). Therefore, many studies focus on the therapeutic potential of HDACi alone or combined with chemotherapeutic drugs for treating different human tumours, such as multiple myeloma, ovarian, gastric, breast, pancreatic and prostate cancers (Prince et al., 2009; Eckschlager et al., 2017; Sun et al., 2018; Autin et al., 2019).

However, no study is available evaluating the anti-cancer activity of any HDACi for treating CMT. Indeed, aberrant activation of HDACs 1, 2, 3, 4 and 6 has been described in breast cancer development and progression (Ediriweera et al., 2019; Zucchetti et al., 2019). Furthermore, a significant relationship has been identified between the expression of HDAC1/3 and the estrogen or progesterone receptor (ER, PR) status and the disease-free survival in the studies by Krusche et al. (2005) and Hu et al. (2009). Additionally, ER and PR-positive cells exert deletion or loss of the HDAC2 gene locus (Krusche et al., 2005; Hu et al., 2009). CMTs share many features, including histopathological and molecular characteristics and clinical outcomes with human mammary tumours (Abdelmegeed and Mohammed, 2018). Epigenetic changes, including DNA methylation, histone modifications and non-coding RNAs, have been found to play significant roles in cancer development by affecting gene regulation. Therefore, recent studies have attracted significant attention, unravelling the role of epigenetic changes in canine tumours (Liu et al., 2014; Xavier et al., 2020; Schabort et al., 2021). Liu et al. (2014) have reported that complex canine mammary carcinomas are associated with several epigenetic dysregulations, including down-regulation of chromatin-modification in about 35 genes and are abnormally enriched with active histone modification H4-acetylation while aberrantly depleted with repressive histone modification H3K9me3a. Schabort et al. (2021) have indicated that novel hyper-methylated intron regions of *ANK2* (encoding ankyrin-2) and *EPAS1* (the gene for endothelial PAS domain protein 1) could potentially be used as tissue biomarkers for CMT. In the study by Brandão et al. (2018), the canine *ESR1* gene, analysed in seventeen CMT samples has been found to be intragenic and non-promoter-associated CpG island methylation. Thus, unlike in humans, *ESR1* is not mediated by DNA methylation in the dog. Due to aberrant activation of HDAC, HDACi might offer therapeutic potential in canine tumours. Some studies have revealed the anti-cancer activity of vorinostat, sulforaphane and trichostatin A as HDACi in canine osteosarcoma cell lines and canine grade 3 mast cell tumours (Nagamine et al., 2011; Rizzo et al., 2017; Eto et al., 2019). In previous studies, these compounds inhibited the viability of cancer cells, induced apoptosis, and suppressed tumour cell invasion. However, a limited number of studies are available in the literature evaluating the epigenetic changes and associated inhibitors in canine cancers. Therefore, we designed the present study to investigate the potential efficacy of NaBu as an HDAC inhibitor in treating CMTs. Our preliminary findings showed that NaBu treatment up to 10 mM did not induce anti-cancer effects on CMT cells. However, particularly 20 mM NaBu significantly suppressed the growth of CMT cells and caused apoptosis. Furthermore, NaBu treatment was more effective in S cells than in C and CS cells. Unlike other CMT subtypes, the estrogen or progesterone expression was not identified in canine mammary sarcomas, which was considered to be associated with the aggressive biological behaviour of these tumours (Dolka et al., 2013). In line with these findings, we may deduce that different NaBu

responses could be mediated by histopathological and molecular characteristics of both human and canine mammary cancers.

Furthermore, NaBu treatment induced apoptosis in CMT cells. However, alterations in the levels of Bax, Bcl-2 and Caspase-3 expression differed in S, C and CS cells. Intriguingly, higher *bcl-2* mRNA and protein levels were detected in S cells after NaBu treatment despite the higher response rates. In the literature, NaBu treatment increased the expression levels of *bax* and *caspase-3* in human colon and gastric cell lines with the down-regulation of *bcl-2* (Wang et al., 2009; Shin et al., 2012). Furthermore, NaBu has been found to cause apoptosis and nuclear damage with the down-regulation of *bcl-2* in the MCF-7 human breast cancer cell line (Wang et al., 2016). Salimi et al. (2017) have reported that NaBu-mediated apoptosis was triggered by increased levels of ROS and caspase activity, with a reduction in mitochondrial membrane potential in the MCF-7 and MDA-MB-468 human breast cancer cells. However, MDA-MB-468 (ER, PR and HER2) triple-negative breast cancer cells are more sensitive to NaBu than MCF-7 cells (Salimi et al., 2017). Therefore, the up-regulation of *bcl-2* in S cells following NaBu administration might have resulted from drug resistance or mechanisms associated with escape from apoptosis. However, there is no study evaluating the anti-cancer activity of NaBu in CMT cells despite similarities to human mammary tumours (Abdelmegeed and Mohammed, 2018). Here, we explored the cytotoxic and apoptotic effects of NaBu on three different CMT cells. Further molecular analyses should be performed to elucidate the underlying molecular mechanisms of the NaBu-induced apoptosis in CMT.

In conclusion, the data presented herein indicate for the first time that NaBu can suppress the proliferation of CMT cells by inducing apoptosis without toxicity in control cells. However, the responses to the NaBu treatment of the different CMT subtypes varied. Therefore, we may conclude that the histological subtype and molecular features might have affected the response to NaBu. Within this context, the molecular classification of CMTs (ER, PR and HER2 status) and the underlying mechanisms of the diverse responses to NaBu should further be investigated at an advanced molecular level.

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