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RESEARCH ARTICLE

The Level of Vasopressin is not solely resulted from the Concentration of Endotoxin but Proportional to Creatinine in Dogs with Pyometra

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ABSTRACT

Pyometra is one of the most common reproductive disorder characterized as fluid accumulation mainly pus and Gram-negative bacteria isolated from uterus in bitches. Impaired function of vasopressin is found in the disease. The impact of the circulating endotoxin concentration on vasopressin involved water regulation is still unclear. To document the effect of endotoxin on vasopressin-involved water regulation in dogs with pyometra, blood samples were collected for examination of circulating endotoxin concentration, osmolarity values, vasopressin concentrations, blood urea nitrogen (BUN), and creatinine. The results indicated that the concentration change of endotoxin was contrary to BUN and creatinine in dogs with pyometra. The trends of concentrations change of BUN and creatinine were similar with osmolarity and vasopressin. Pyometric dogs with high level of circulating endotoxin had significantly lower ($P<0.05$) plasma osmolarity and vasopressin concentration than those which had low levels of serum endotoxin. Plasma osmolarity was positive correlated with both BUN and creatinine concentrations ($rP=0.55$ and 0.53, respectively; $P<0.01$). Likewise, the vasopressin concentration showed good correlation with serum creatinine value ($rP=0.73$; $P<0.05$). Pyometric dogs with high levels of BUN (> 20 mg/dL) and creatinine (> 1.5 mg/dL) also had plasma vasopressin significantly higher ($P<0.05$) than those with low levels of BUN and creatinine and control dogs. The overall results suggested that elevated vasopressin was not mainly associated with the circulating endotoxin concentration and some other factors may collaborate with endotoxin causing impaired function of vasopressin.

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INTRODUCTION

Pyometra is a hormone-directed diesteral disease commonly observed in older or middle-aged intact female dogs (Niskanen and Thrusfield, 1998; Fukuda 2001; Sugiura *et al*., 2004). Until now, *Escherichia coli* is considered to be the main causative pathogen of dogs with pyometra (Wadas *et al*., 1996; Dhaliwal *et al*., 1998). The release of endotoxin from lysed *E. coli* cells will cause dramatic adverse effects on dogs with pyometra (Stone *et al*., 1988; Okano *et al*., 1998).

Among clinical signs of dogs with pyometra, abnormal water regulation related polyuria, polydipsia, and hyposthenuria are commonly observed in clinics (Stone *et al*., 1988; Heiene *et al*., 2004). Continuous polyuria can induce the increment of plasma osmolarity and further stimulates vasopressin secretion (Robertson and Athar, 1976; Bush, 1988). Though the etiology of canine pyometra disease is well studied, impaired water regulation is still questionable. Based on the etiology of the primary causes of polyuria and polydipsia (PU/PD), secondary nephrogenic diabetes insipidus is presumptive in any dogs with pyometra because of increased plasma vasopressin is found (Feldman and Nelson, 1989; Heiene *et al*., 2004). Interfering with vasopressin binding or aquaporin-2 (AQP_2) trafficking at distal and collecting tubules in patient with nephrogenic diabetes insipidus resulted in the defect of urinary concentrating ability (Kotnik *et al*., 2007). Translocation of AQP_2 from intracellular vesicles into the plasma membrane will result

in strong increase of water permeability and accelerated reabsorption of water from primary urine (Nielsen *et al*., 1995). Endotoxin was ever suspected as the factor causing secondary nephrogenic diabetes insipedus in dogs with pyometra (Feldman and Nelson, 1989), but there is still not proved until now. Further elucidation of the causes behind the elevated vasopressin value and PU/PD in dogs suffer from pyometra is thus a prominent issue.

Even though only limited information was available regarding why vasopressin was elevated in dogs with pyometra, high level of gram-negative originated endotoxin seems to be one prominent risk factor (Battaglia *et al*., 1998). A number of researchers have evaluated the influence of endotoxin induced endotoxemia on magnocellular neurons and their effect on vasopressin secretion, but variable results are shown in different experimental designs (Kasting *et al*., 1985; Parrott *et al*., 1997; Battaglia *et al*., 1998; Grinevich *et al*., 2001 & 2003). Beside affect vasopressin, endotoxin will decrease glomerular rate and subsequent creatinine clearance (Hinshaw *et al*., 1961). In fact, the glomerular filtration rate can be indirectly measured through monitoring circulating creatinine (Finco *et al*., 1995). However, the role of endotoxin on the change of serum creatinine and BUN in dogs with pyometra was limited. Indeed, serum creatinine and BUN help account for not only the effect of endotoxin on glomerular filtration but also the hydration status modulated by vasopressin (Marcia and Kubo, 1975; Finco and Duncan, 1976).

Though a large number of studies have been made on the pathogenesis of pyometra in dogs, little is known about vasopressin involved water regulation, especially the interaction of vasopressin and endotoxin in dogs with pyometra. This paper is intended to investigate the effects of endotoxin on plasma osmolarity and vasopressin levels and elucidate the relationship between serum metabolites, osmolarity and vasopressin during water regulation process in dogs with pyometra.

MATERIALS AND METHODS

Animals

Eleven female dogs were diagnosed as suffering from pyometra at Veterinary Medicine Teaching Hospital, National Chung Hsing University, Taichung, Taiwan, were used for the study. The mean age was 10.4±0.5 years (from 8 to 13 years old). Preliminary diagnosis was based on the history, clinical signs, radiography and ultrasonography. The final diagnosis was made by gross observation after ovariohysterectomy and bacteriological examination. The most common clinical signs of dogs suffered from pyometra were depression, fever, anorexia, and vomit. Eight dogs with normal physical status for ovariohysterectomy were assigned as control group. The sample using in this study was approved by owner's oral consent.

Anaesthetic protocols

General anaesthesia in all control bitches and 11 of the pyometra bitches was induced with intravenously administered propofol (Lipuro®, B. Braun Melsungen, German) and maintained by inhalation anaesthesia with isoflurane (IsoFlo®, Abbott, Farum, USA) and O_2. Intravenous fluid therapy (Dextrose-lactated Ringer's solution, Taiyu, Taiwan) was administered to all pyometra cases and controls during surgery.

Measurements of Plasma Osmolarity and Vasopressin Concentration

Blood samples were collected from jugular vein before surgery and stored in heparin-coated tubes (Vacuette®, Cen-Med Enterices, USA). After centrifugation, plasma samples for the measurements of osmolarity and vasopressin were collected and stored at -70°C. Plasma osmolarity was measured by micro osmometer (Advanced™ micro osmometer model 3300, Massachusetts, USA). Plasma vasopressin values were measured by radioimmunoassay (EURIA-Vasopressin®, Euro-Diagnostica, Malmö, Sweden).

Serum Endotoxin Examination

The blood was stored in endotoxin-free polystyrene tube in 4°C refrigerator for 15 min let blood clotting; after centrifugation, the serum samples were stored at -70°C. All instruments and containers involved in endotoxin examination were endotoxin free. The concentration of serum endotoxin was quantified by kinetic tubidimetric method using the Limulus coagulation enzyme (ES test, Wako chemicals, Virginia, USA). Briefly, 0.1 ml plasma was diluted with 0.9 ml of 0.02% Triton X-100 and heated at 70℃ for 10 minutes. After sample pretreatment, 0.1 ml of diluted plasma samples were taken and added to the plastic container coated with *Limulus* amebocyte lysate at bottom. The turbidity of each sample was measured at specific time by Toxinnmeter (ET-2000J, Wako Pure Chemical Industrial, Japan) under 37°C. The endotoxin concentrations were calculated by comparison with the standard value provided by the manufacture. To clarify if circulating endotoxin affect vasopressin level in dogs with pyometra, the dogs were subdivided into two groups, higher and lower concentration of endotoxin groups.

Serum Metabolites Measurement

Blood samples were taken as described previously. Blood chemistry was focus on serum metabolites, including blood urea nitrogen (BUN) and creatinine. All serum metabolites analyses were performed using Ciba-Corning Express Plus Serum Analyzer (Massachusetts, USA). To investigate if serum metabolites could be possible parameters for evaluation of vasopressin changes, pyometra dogs were subdivided into two groups based on their serum metabolites levels. The cut-off values were set according to the top normal range in dog (Creatinine > 1.5 mg/dL and BUN > 20 mg/dL) (Bush, 1991). In this study, creatinine concentrations higher than 1.5 mg/dL and BUN higher than 20 mg/dL was denoted as high metabolites group (n = 4) while the others were denoted as low metabolites group (creatinine < 1.5 mg/dL and BUN < 20 mg/dL, n = 7).

Statistical Analysis

Statistical analysis was performed using SAS 9.0 (SAS Institute Inc., Cary, NC, USA). Differences in plasma vasopressin, osmolarity, and endotoxin concentrations between control and pyometra dogs with

different levels of serum metabolites were assessed by Mann-Whitney U test. For all comparisons, P value < 0.05 was considered as significant difference. All data presented in the study were mean ± S.E.M. Correlation coefficient (rP) was calculated for plasma osmolarity, vasopressin concentrations, endotoxin concentration, and serum metabolites (BUN and creatinine) by Spearman correlation.

RESULTS

In the blood examination, average WBC value was 3.6±0.6 M/mm^3 (ranged from 1.3 M/mm^3 to 7.1 M/mm^3) in dogs with pyometra and was 1.3±0.1 M/mm^3 (ranged from 0.7 to 1.7 M/mm^3) in control dogs (n = 8). The mean serum creatinine concentration in dogs with pyometra was 1.7±0.5 mg/dL (ranged from 0.3 to 5 mg/dL) and the mean BUN concentration was 55.4±19.8 mg/dL (ranged from 11 to 221 mg/dL). Both serum metabolites in dogs with pyometra tended to be higher than the control ones but did not reach significant difference. However, serum creatinine and BUN in pyometra with low endotoxin group were both significantly higher than those with high endotoxin group (P<0.05) (Fig. 1).

There was no significant difference in plasma osmolarity between pyometra (295.3±8.5 mOsm/kg) and control (308.0±3.0 mOsm/kg) dogs. After grouping the dogs with pyometra by serum metabolites, plasma osmolarity in high metabolites group (n=4) tended to be higher than that of the low metabolites group (n=7) but without significant difference. Plasma osmolarity in low metabolites group was significantly lower than that of control (Fig. 2). Moreover, plasma osmolarity was correlated with both BUN and creatinine concentration (rP = 0.55 and 0.53, respectively; P<0.01). The parallel effect was found between plasma osmolarity and serum metabolites.

The mean plasma vasopressin concentration in dogs with pyometra was 8.8±3.1 pg/mL (ranged from 2.5 to 34.6 pg/mL), whereas the mean vasopressin in control dogs was 3.8±0.8 pg/mL (range from 1.4 to 9.9 pg/mL). Vasopressin concentration in dogs with pyometra was higher than that in control but without significant difference. Plasma vasopressin concentration in high metabolites group (17.3±6.9 pg/mL) was significantly higher (P<0.05) than that in low metabolites group (4.0±0.7 pg/mL) (Fig. 3). Significant correlation (rP = 0.73; P<0.05) was found between plasma vasopressin concentration and serum creatinine (Fig. 4) value but not significantly correlated with BUN value.

The serum endotoxin concentration in dogs with pyometra (0.96±0.43 ng/mL, from 0.004 to 5.03 ng/mL) was significantly higher than the controls (0.01±0.01 ng/mL). Four dogs with pyometra were denoted as low endotoxin group, the distribution of endotoxin concentration was from 0.004 to 0.07 ng/mL (0.03±0.02 ng/mL). There had no significant difference of the endotoxin concentration between control and pyometra with low endotoxin group (P=0.26). The other seven dogs with pyometra were denoted as high endotoxin group, the distribution of endotoxin concentration was from 0.57 to 5.03 ng/mL (1.49±0.60 ng/mL). Pyometra with high endotoxin group had significantly higher endotoxin than

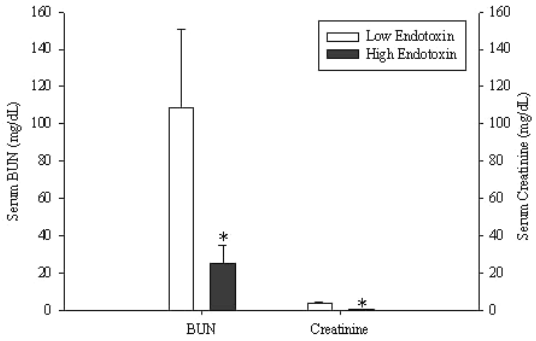

Fig. 1: Serum endotoxin concentration in dogs with pyometra with high (n = 7) and low (n = 4) level of serum metabolites, *, P<0.05 compared to low level of endotoxin group.

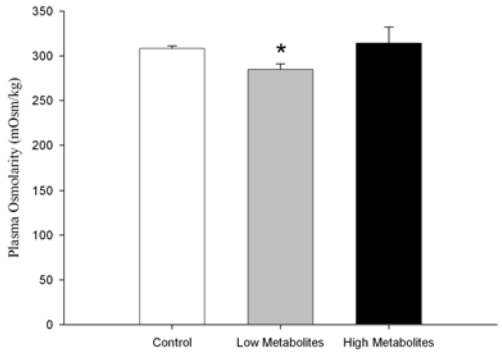

Fig. 2: The averaged osmolarity of control and pyometra dogs with subdivisions according to the concentrations of serum metabolites: high metabolites (creatinine > 1.5 mg/dL, BUN > 20 mg/dL, n = 7), and low metabolites (creatinine < 1.5 mg/dL and BUN < 20 mg/dL, n = 4).

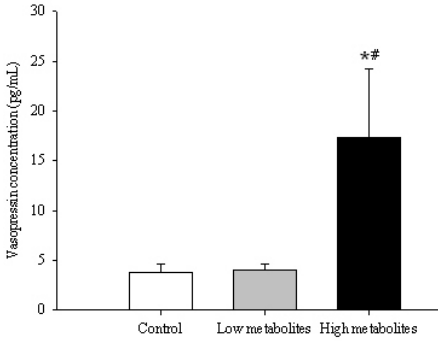

Fig. 3: The averaged vasopressin of control and pyometra dogs with high serum metabolites and low serum metabolites. *, P<0.05 compared to control; #, P<0.05 compared to pyometra with low level of serum metabolites.

that with low endotoxin (P<0.01) and control (P<0.01) groups. The vasopressin value of pyometra dogs with higher circulating endotoxin concentration (4.0±0.7 pg/mL) was significantly lower than that with lower circulating endotoxin concentration (17.3±6.9 pg/ml) (Fig. 5A). This result coincided and connected with the result of Fig. 1 and Fig. 4. In parallel with the vasopressin result, the plasma osmolarity in pyometra dogs with higher circulating endotoxin concentration (287.0±2.8 mOsm/kg) was also significantly lower than that with lower circulating endotoxin concentration (328.5±15.2 mOsm/kg) (Fig. 5B).

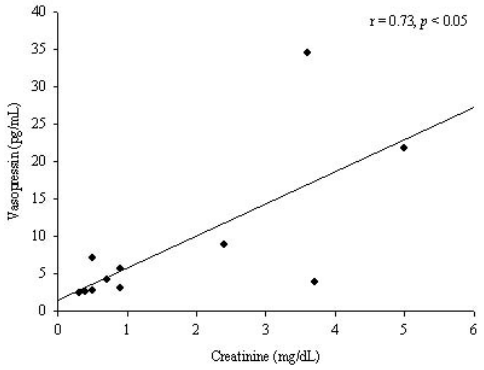

Fig. 4: The correlation between plasma vasopressin and serum creatinine level in dogs with pyometra.

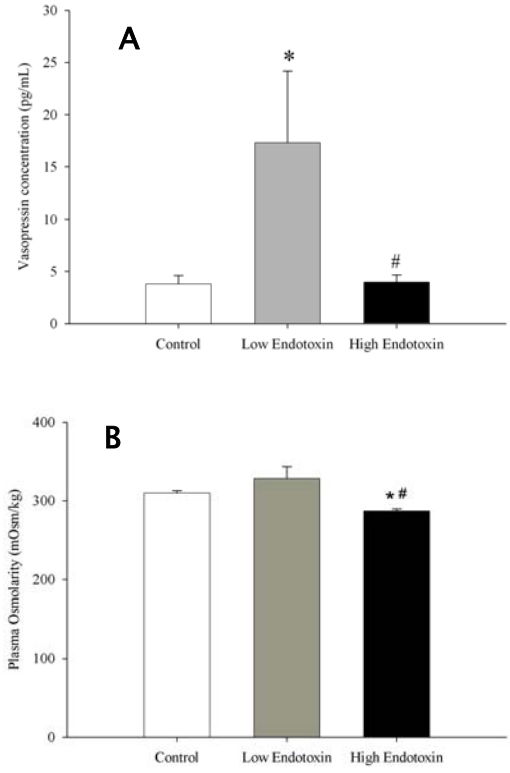

Fig. 5: The effect of endotoxin on the change of secretion of vasopressin (upper) and plasma osmolarity (lower) in dogs with pyometra. A) Comparison of averaged vasopressin concentration between control (n = 8) and pyometra dog were further subdivided according to the mean endotoxin concentration (0.01 ng/mL) of control dogs. *P<0.05 compared to control; #P<0.05 compared to pyometra with low level of circulating endotoxin.

DISCUSSION

Based on our knowledge, beside pyometra disease, impaired water regulation is rare observed in other bacterial infection (Massol *et al*., 1985) or female reproductive diseases (Greco, 2007). It is interesting that dogs with bacterial infection in the uterus have PU/PD. Until now, impaired function of vasopressin is revealed in this (elevated concentration of vasopressin in dogs with pyometra) and previous study (Heiene *et al*., 2004). The possible role of endotoxin on vasopressin involved water regulation was also proposed in the study. The opposite change between serum metabolites (BUN and creatinine) and endotoxin was first observed in our study. Increased serum metabolites might be ascribed to dehydration in dogs with pyometra; however, the endotoxin was not parallel increased but decreased in dehydration status. Besides, the osmolarity of pyometra group with higher serum metabolites (P-HSM) was not significantly higher than control. This might explained the pyometra group with higher serum metabolites was not suffering with severe dehydration. In homeostasis, elevated osmolarity is the main trigger to stimulate vasopressin releasing (Robertson and Athar, 1976; Bush, 1988). In our result, though the osmolarity of P-HSM was not significantly higher than control, vasopressin was significantly higher than control. This could be because of the decreased threshold of osmolarity to stimulate vasopressin release in dogs with pyometra (Heiene *et al*., 2004). Together with the relationship between serum metabolites, osmolarity, and vasopressin, the concentrations change of these factors were in the same trend. Significant correlation of vasopressin and creatinine again demonstrated the trend. Accompany with the result of opposite trend of endotoxin and serum metabolites, pyometra group with low endotoxin group should be had higher osmolarity and vasopressin level. This result was also demonstrated in our study.

Based on our observation of the vasopressin change contrary to endotoxin, the question then arises concerning the reasons behind the elevated vasopressin concentration. Actually, high levels of serum endotoxin could be one feasible factor but not the only factor resulting in elevated plasma vasopressin in dogs with pyometra (Kasting *et al*., 1985; Grinevich *et al*., 2001). The real reason was unclear, but it may be ascribed to another factor which can stimulate the secretion of vasopressin. In fact, besides the direct stimulation of vasopressin secretion by endotoxin, indirect stimulation by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is also found in rats (Bojanowska and Guzek, 1989). The increase of prostaglandin expression is resultant from endotoxin in rat uterus (Ribeiro *et al*., 2004), and this was similar in cow, bitch and ferret (Heap, 1975). Refer to the study of Silva and colleagues, prostaglandin synthesis enzymes are all up-regulated and subsequent PGE_2 and $PGF_{2\alpha}$ content significantly higher in canine pyometra than control (Silva *et al*., 2009, 2010). The PGE_2 not only exerts strong anti-inflammation effect but also counteracts the VP-induced water permeability in renal collecting ducts (Strassmann *et al*., 1994; Kambayashi *et al*., 1995). VP influences water reabsorption by binding with V2-receptor at basolateral membrane of distal and collecting tubules of the kidney to trigger the subsequent signal transduction pathways involving aquaporin-2 (AQP_2) trafficking (Kamsteeg *et al*., 2000). AQP_2 expression at cell surface has been proved to be involved in depolymerization of actin network; on the contrary, increased actin polymerization will result in blockage of VP-induced AQP_2 translocation (Klussmann *et al*., 2001). When PGE_2 binds to PGE_2 receptor type 3 in collecting duct, VP-

induced AQP_2 translocation was blocked (Tamma *et al*., 2003). Hence, VP involved water reabsorption is inhibited and result in impaired function of water regulation. In all, prostaglandins may play an important role in the regulation of vasopressin in addition to endotoxin in canine pyometra (Klussmann *et al*., 2001; Tamma *et al*., 2003; Silva *et al*., 2010).

The result of the study indicated lower plasma osmolarity and vasopressin concentration in pyometra dogs with high levels of circulation endotoxin, these findings may be ascribed to the less effect from new endotoxin stimulation. Indeed, it has been evidenced that dogs had suffered from endotoxemia for a period of time, the vasopressin concentration probably would not increase while exposed to new endotoxin stimulation (Grinevich *et al*., 2001; Grinevich *et al*., 2003). According to the mentioned above, thereafter, we hypothesized that in the early stage of pyometra, high levels of $PGF_{2\alpha}$ should be found in endometrium and circulation but low or undetectable levels of endotoxin. The concentration of endotoxin or the time of endotoxin affect endometrial cells could be not sufficient to switch from $PGF_{2\alpha}$ to PGE_2. During this period, the plasma vasopressin concentration would increase via indirect activation by $PGF_{2\alpha}$, this might explain why significantly higher plasma vasopressin concentrations could be found in pyometra cases with low levels of circulating endotoxin. As the disease developing, the level of plasma vasopressin concentration was dependent on the equilibrium of $PGF_{2\alpha}$ and PGE_2 and the concentration of circulation endotoxin. In this period, the circulating endotoxin would get higher because more and more bacteria were killed. Regardless of the increase in circulating PGE_2 or endotoxin, the AQP_2 expression and translocation will be affected and resulted in subsequent polyuria. In the late stage of pyometra, high levels of circulation PGE_2 and endotoxin would be found. Unfortunately, the endometrium or plasma $PGF_{2\alpha}$ and PGE_2 were not examined in our study; nevertheless, we still have provided a possible alternative way to investigate the mechanism involving the regulation of vasopressin secretion.

In conclusion, the current study was first investigated the relationship between the circulating endotoxin and vasopressin concentrations in relation to water regulation in dogs with pyometra. The concentration change of serum metabolites, osmolarity, and vasopressin was in the same trend but in the opposite with endotoxin. Endotoxin was not the main cause of elevated plasma vasopressin in dogs with pyometra. Serum creatinine was found to be proportional to vasopressin level; through measuring serum creatinine, the degree of elevated vasopressin could be estimated. The study provided valuable information concerning the reason of elevated plasma vasopressin concentration in dogs with pyometra.

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