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Effect of Orexin Infusion into Third Ventricle on the Metabolic Parameters in the Goats Fed Low Energy Levels in Diets

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Abstract: The goal of this study was to determine whether orexin affects the mean plasma concentrations of metabolic parameters such as thyroxine (T4), triiodothyronine (T3), Growth Hormone (GH), insulin, glucagon, glucose, fatty acid and urea in the goats fed different energy content in diets. Sixteen goats were randomly divided into 4 groups. Animals in group 1 and 2 were fed 100% and animals in groups 3 and 4 were fed 50% energy content in diet for 20 days. After 20 days, animals in groups 1 and 3 received daily infusion of 1 μg orexin and groups 2 and 4 received daily infusion of 2 μg orexin into their third ventricle for 5 days. Lower dietary energy intake and infusions of 1 and 2 μg orexin significantly ($p<0.01$) decreased the mean plasma concentrations of T3, T4, insulin and glucose and significantly ($p<0.01$) increased the mean plasma concentrations of GH, glucagon, fatty acid and urea of the animals in groups 3 and 4. The results of this experiment indicated that orexin may negatively affect the T3, T4, insulin and glucose and increase GH, glucagon, fatty acid and urea in the goats with negative energy balance, but not in those with the positive energy balance.

Key words: Orexin, metabolic hormones, goat

INTRODUCTION

Orexin is a 33-amino-acid neuropeptide that is mostly found in hypothalamus (Antunes *et al*., 2001; Arihara *et al*., 2000; Backberg *et al*., 2002). Based on its neuron distributions in hypothalamus, orexin coexists with many other neurons. For example, in hypothalamic area, orexin coexists with neurons secreting different neurotransmitters such as GHRH, GABA, noradrenaline, 5-hydroxytryptamine (5-HT) and NPY (Al-Barazanji *et al*., 2001; Balasko *et al*., 1999). Therefore, orexin controls different physiological actions on many different tracts (Antunes *et al*., 2001; Al-Barazanji *et al*., 2001; Arihara *et al*., 2000; Backberg *et al*., 2002; Balasko *et al*., 1999; Ehmke and Just, 2003). One of the physiological actions is its effect on metabolism and feeding behaviors that make orexin as an orexinergic hormone (Backberg *et al*., 2002). The orexigenic effect of orexin decrease or increase plasma levels of insulin, glucagon, somatostatin, gastrin and increase the release of growth hormone (Hagan *et al*., 1999; Lopez *et al*., 2004; Meghan and Samson, 2003; Mitsuma *et al*., 1999; Nowak *et al*., 2005; Russell *et al*., 2002). Most of the above studies were conducted in human and rat as a nonruminant. Ruminants have different metabolism from that of nonruminants (Harrison and Leat, 1975). It is assumed that the control of feeding behavior is different from that of nonruminants. There are very few reports about the orexigenic effect of orexin on the metabolic hormones in ruminants fed different energy content in diet. Therefore, the first goal of this experiment was to determine whether orexin affects the mean concentrations of metabolic parameters in the goats fed different energy content in diets.

Among many studies done on the effect of orexin on metabolic hormone, there are no reports about the effect of the orexin on thyroid hormones under different energy intake. The importance of thyroid hormones in metabolism is well known. For example, thyroid hormones play an important role in the regulation of energy homeostasis via oxygen consumption and heat generation (De Jesus *et al*., 2001; Lanni *et al*., 2001). Changes in basal metabolic rate caused by different energy content in diet is accompanied by changes of thyroid hormones secretions. Therefore, the second goal of this study was to determine whether orexin alters the thyroid hormones secretion in the goats fed different energy content in diet.

MATERIALS AND METHODS

Experimental design: This experiment were conducted during 2007-2009. Sixteen goats (weighing between 40 to 50 kg) were randomly divided into 4 groups and kept in Animal Science Research Institute of Karaj. Animals in group 1 and 2 were fed 100% energy (NE) and animals in group 3 and 4 were fed 50% energy (LE) content in diet for 20 days. Gross energy and chemical compositions of

Table 1: Experimental rations and prepared energy and nutrients

	Energy (%)	
Diet	100	50
Ingredients/nutrition		
Wheat straw (g day^{-1})	10.00	260.00
Alfalfa (hay) (g day^{-1})	50.00	50.00
Corn (grain) (g day^{-1})	10.00	220.00
Corn gluten meal (g day^{-1})	210.00	85.00
Bone meal (g day^{-1})	1.34	0.47
Salt (g day^{-1})	1.66	1.22
Magnesium oxide (g day^{-1})	0.69	-
Vitamin and mineral supplement	3.50	3.50
Metabolizable energy (MJ kg^{-1})	13.03	9.73
Crude protein (%)	42.00	13.72
Calcium (%)	0.52	0.24
Phosphorous (%)	0.52	0.24
Sodium (%)	0.45	0.21
Magnesium (%)	0.24	0.11
Dry mater intake (g day^{-1})	287.00	620.00
Metabolizable energy intake (MJ kg^{-1})	3.74	6.03
Metabolizable protein intake (g day^{-1})	56.00	55.37

feedstuffs consisted of dry mater, crude protein, crude fiber, ether extract, total ash, NDF, ADF, calcium and phosphorous were analyzed in the Animal Science Research Institute of Karaj during 2007-2009. Diets were formulated based on (Agricultural and Food Research Council, 1995) (Table 1). We chose this formulation based on the Shahid Beheshti University permission. During the course of the experiment, daily feed was weighed based on body weight and individually given to each goat every morning. The goats had free access to fresh water. Diet 1 and 2 were consisted 100 and 50% of maintenance energy requirements, respectively. Other requirements were balanced at maintenance level. After 20 days, all animals were prepared for surgery. Goats were anesthetized throughout the surgery for third ventricle cannulation under stereotaxic methods and jugular vein cannulations. Surgical procedures were done under general anesthesia induced by sodium pentobarbital and maintained by halothane in a closed circuit system (Khazali, 1992). Each goat was kept in a single cage for a 4 days recovery period. During recovery period, cannules were washed by PBS solution to prevent from clotting. After surgery, on day 5, goats in group 1 and 3 received 1 μg orexin and goats in group 2 and 4 received 2 μg orexin into their third ventricles for 5 days. Body weight of animals was measured on day 1 and 20 of the experiment.

Blood collection: Blood samples were collected from cannules that were put into the jugular veins, everyday from 4 days before first infusion of orexin until 4 days after the last orexin infusion. Blood samples were kept at 4°C until centrifugation. A saturated sodium citrate solution (40 μL sodium citrate solution mL^{-1} blood) was added to the samples before centrifugation to prevent clotting of plasma during storage. Plasma was stored at -20°C until assayed for T3, T4, insulin, GH, glucagon, glucose, fatty acid and urea.

Hormone assays: Plasma T3, T4, insulin, GH and glucagon were measured by a homologous double-antibody radioimmunoassay (RIA). For GH assay, ovine GH (TYN-OG) and antisera against GH were provided by Tabeshyarnoor Co.(Industerial City of Bu-Ali, Hamadan, Iran). Ovine GH (TYN-OG) was used for iodination. A seven-point standard curve ranging from 0.04 to 10 ng GH was used. An average assay binding of 40% was achieved using an initial 1:20000 dilution of GH antiserum for GH assays. The inter- and intra-assay variations were 6 and 9%, respectively. For insulin assay, ovine insulin (TYN-OI) and antibody against insulin were provided by Tabeshyarnoor Co. (Induterial City of Bu-Ali, Hamadan, Iran). Ovine insulin (TYN-OI) was used for iodination. A seven-point standard curve ranging from 0.02 to 10 ng insulin was used. An average assay binding of 30% was achieved using an initial 1:5000 dilution of insulin antiserum for insulin assays. The inter- and intra-assay variations were 8 and 5%, respectively. For glucagon assay, human glucagon (TYN-HC) and antibody against glucagon were provided by Tabeshyarnoor Co. (Industerial City of Bu-Ali, Hamadan, Iran). Human glucagon (TYN-HC) was used for iodination. A seven-point standard curve ranging from 0.02 to 10 ng insulin was used. An average assay binding of 35% was achieved using an initial 1:10000 dilution of glucagon antiserum for glucagon assays. The inter- and intra-assay variations were 7 and 6% respectively. For T3 assay, T2 was purchased from Sigma Chemical Company and T3 antisera were purchased from Chemicon Co. (Temmecula, Ca). The T2 was used for iodination. A six-point standard curve ranging from 0.32 to 5.2 ng T3 mL^{-1} was used. An average assay binding of 70% was achieved using an initial 1:5000 dilution of T3 antiserum for T3 assays. The inter- and intra-assay variations were 7 and 7%, respectively. For T4 assay, T3 was purchased from sigma chemical company and T4 antisera was purchased from Chemicon Co. (Temmecula, Ca). The T3 was used for iodination. A six-point standard curve ranging from 2.2 to 25 ng T4 mL^{-1} was used. An average assay binding of 60% was achieved using an initial 1:5000 dilution of T4 antiserum for T4 assays. The inter- and intra-assay variations were 7 and 5%, respectively. For glucose assay, ELISA kits were purchased from sigma chemical company. A six-point standard curve ranging from 20 to 250 mg glucose dL^{-1} was used. An average assay binding of 35% was achieved. The inter- and intra-assay

variations were 4 and 6%, respectivity. For fatty acid assay, ELISA kits were purchased from Sigma Chemical Company. A six-point standard curve ranging from 10 to 150 mg fatty acid dL^{-1} was used. An average assay binding of 45% was achieved. The inter- and intra-assay variation were 5 and 8%, respectively. For urea assay, ELISA kits were purchased from sigma chemical company. A six-point standard curve ranging from 10 to 150 mg urea dL^{-1} was used. An average assay binding of 32% was achieved. The inter- and intra-assay variations were 4 and 6%, respectively.

Statistical analysis: All analysis were conducted using General Linear Model procedures (SAS, 1996). Data were analyzed using an analysis of variance for a repeated measure design. Mean comparisons were evaluated by least significant difference with single degree of freedom.

RESULTS

T3 and T4: Infusions of 1 and 2 μg orexin into third ventricle significantly ($p<0.01$) decreased mean plasma concentrations of T3 , but not T4 of the animals in groups 1 and 2 that were fed NE. Mean plasma T3 levels of the animals in group 1 and 2 were about 2.5, 1.8, 2.2 and 2.4, 1.7, 2.1 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 1). Also, Mean plasma concentrations of T4 of the NE animals in group 1 and 2 were about 41, 40, 40 and 39, 42, 41 ng mL^{-1} before, during and after infusion of orexin respectively (Fig. 2). Plasma T3 and T4 levels of LE fed animals in groups 3 and 4 were significantly ($p<0.01$) lower than that of the NE fed animals (Fig. 1, 2). Orexin infusions significantly ($p<0.01$) decreased plasma T3 and T4 levels in the LE fed animals (Fig. 1, 2).

GH: Low energy content in diet increased the GH plasma levels of the animals in group 3 and 4 in comparison with plasma GH levels of those animals fed NE. Further to the effect of lower energy dietary intake, infusions of 2 μg orexin significantly ($p<0.01$) increased the mean plasma GH levels in the animals of group 4 (from 3 to 5), followed by declining GH level from 5 to 4.2 after infusion of orexin. Infusions of 1 μg orexin did not change the mean plasma concentrations of the GH in the animals of group 1 and 2 that fed 100% energy content in diets for 20 days. Mean plasma concentrations of the GH of group 1 were about 1.9, 1.8 and 1.8 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 3). Two micrograms orexin did not change the mean plasma concentrations of the GH in the animals of group 2 that were fed NE. Mean plasma concentrations of the GH of group 2 were about 1.8, 1.7 and 1.7 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 3).

Insulin: Infusions of 1 and 2 μg orexin did not change the mean plasma concentrations of the insulin in the animals of groups 1 and 2 that were fed NE. Mean plasma concentrations of the insulin of the animals in groups 1 and 2 were about 39, 40, 44 and 38, 38, 44 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 4).

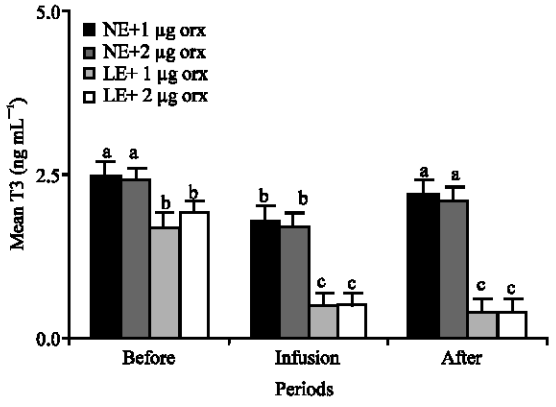

Fig. 1: Mean plasma concentrations of T3 of the animals in the different groups of 1 (NE and 1 μg orexin), 2 (NE and 2 μg orexin), 3 (LE and 1 μg orexin) and 4 (LE and 1 μg orexin) and before, during and after infusions of ghrelin. NE: Normal energy; LE: Low energy. Treatments with different letter(s) are different at $p<0.01$

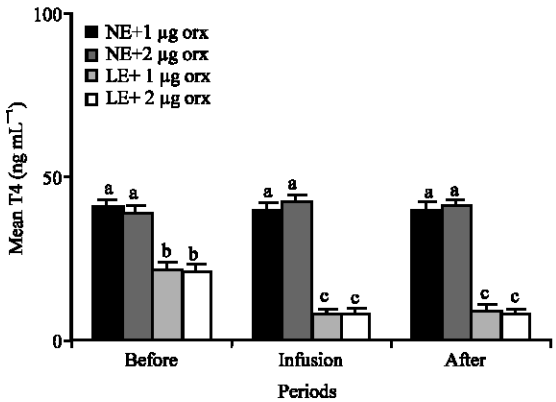

Fig. 2: Mean plasma concentrations of T4 of the animals in the different groups of 1 (NE and 1 ug orexin), 2 (NE and 2 ug orexin), 3 (LE and 1 ug orexin) and 4 (LE and 1 ug orexin) and before, during and after infusions of ghrelin. NE: Normal energy; LE: Low energy. Treatments with different letter(s) are different at $p<0.01$

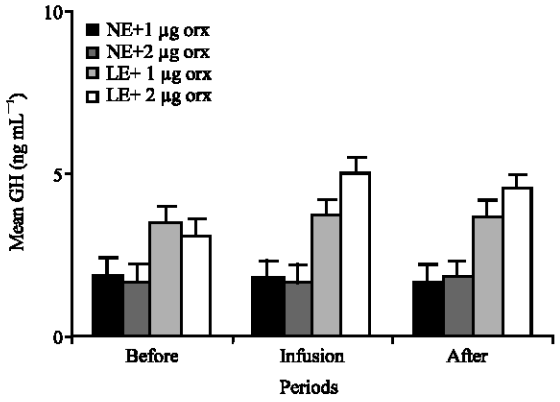

Fig. 3: Mean plasma concentrations of GH of the animals in the different groups of 1 (NE and 1 ug orexin), 2 (NE and 2 µg orexin), 3 (LE and 1 ug orexin) and 4 (LE and 1 µg orexin) and before, during and after infusions of ghrelin. NE: Normal energy; LE: Low energy

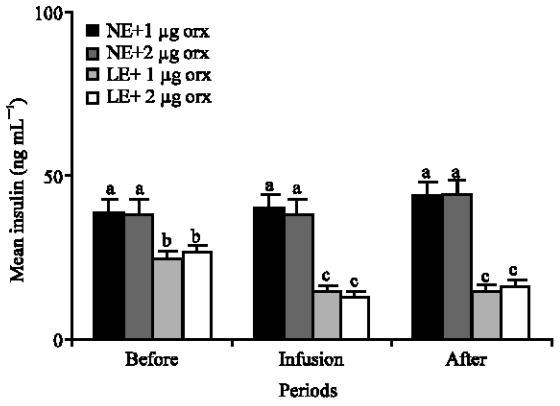

Fig. 4: Mean plasma concentrations of insulin of the animals in the different groups of 1 (NE and 1 µg orexin), 2 (NE and 2 µg orexin), 3 (LE and 1 ug orexin) and 4 (LE and 1 µg orexin) and before, during and after infusions of ghrelin. NE: Normal energy; LE: Low energy. Treatments with different letter(s) are different at $p<0.01$

Mean plasma concentrations of insulin of the animals in groups 3 (25 ng mL^{-1}) and 4 (27 ng mL^{-1}) fed LE were significantly ($p<0.01$) lower than the plasma insulin levels of those animals in groups 1 (39 ng mL^{-1}) and 2 (38 ng mL^{-1}) fed NE (Fig. 4). Infusions of 1 µg orexin significantly ($p<0.01$) decreased the mean of the plasma levels of insulin in the animals of group 3 from 25 to 15. Also, mean of the plasma levels of insulin of the animals in group 4 significantly ($p<0.01$) decreased from 27 to 13 by infusion of 2 µg orexin (Fig. 4).

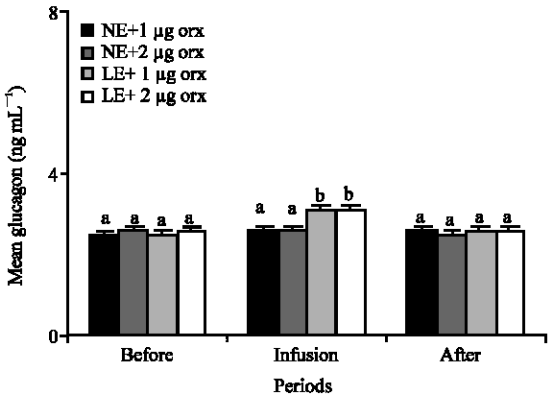

Fig. 5: Mean plasma concentrations of glucagon of the animals in the different groups of 1 (NE and 1 µg orexin), 2 (NE and 2 ug orexin), 3 (LE and 1 µg orexin) and 4 (LE and 1 ug orexin) and before, during and after infusions of ghrelin. NE: Normal energy; LE: Low energy. Treatments with different letter(s) are different at $p<0.01$

Glucagon: Orexin infusions did not change the mean plasma concentrations of the glucagon in the animals of group 1 and 2 that were fed NE. Mean plasma levels of the glucagon of the animals in group 1 and 2 were about 2.5, 2.6, 2.6 and 2.6, 2.6. 2.5 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 5). Infusions of 1 µg orexin significantly ($p<0.01$) increased the mean of plasma levels of orexin in the animals of group 3 from 2.6 to 3.1, followed by decreasing of glucagon plasma levels from 3.1to 2.6 after infusion of orexin. Also, mean of plasma levels of glucagon of the animals in group 4 significantly ($p<0.01$) increased from 2.5 to 13.1 by infusion of 2 µg orexin (Fig. 5).

Glucose: Orexin did not change the mean plasma concentrations of the glucose of the animals in group 1 and 2 that were fed NE. Plasma concentrations of the glucose of groups 1 and 2 were about 50, 48, 50 and 52, 50, 50 mg dL^{-1} before, during and after infusion of orexin, respectively (Fig. 6). Plasma glucose levels of the LE fed animals in groups 3 (42 mg dL^{-1}) and 4 (41 mg dL^{-1}) were significantly ($p<0.01$) lower than the mean plasma concentrations of glucose of those animals in the group 1 (50 mg dL^{-1}) and 2 (52 mg dL^{-1}) fed NE (Fig. 4). Infusions of 1 and 2 µg orexin significantly ($p<0.01$) decreased the glucose levels among those animals of group 3 and 4 fed LE (Fig. 6).

Fatty acid: Infusions of 1 and 2 µg orexin significantly ($p<0.01$) increased the mean plasma concentrations of the fatty acid of the animals in groups 1 and 2 that were fed

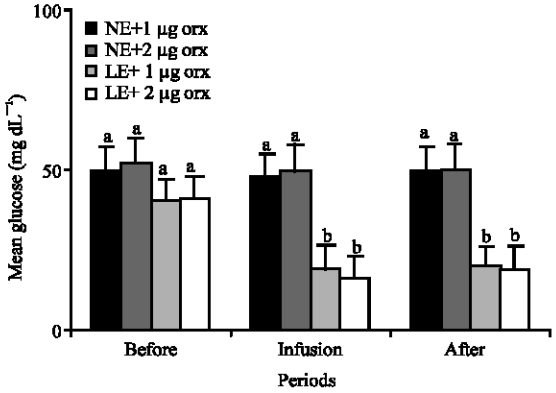

Fig. 6: Mean plasma concentrations of glucose of the animals in the different groups of 1 (NE and 1 µg orexin), 2 (NE and 2 µg orexin), 3 (LE and 1 µg orexin) and 4 (LE and 1 µg orexin) and before, during and after infusions of ghrelin. NE: Normal energy; LE: Low energy. Treatments with different letter(s) are different at $p<0.01$

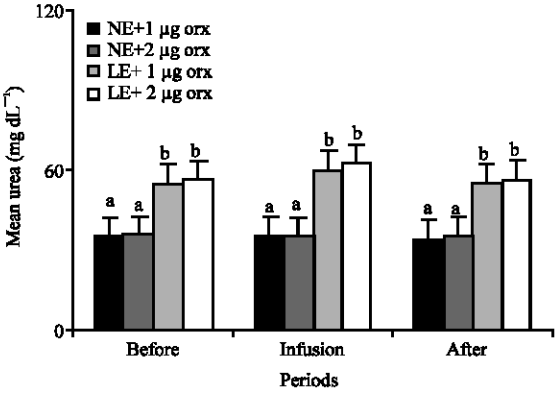

Fig. 8: Mean plasma concentrations of urea of the animals in the different groups of 1 (NE and 1 µg orexin), 2 (NE and 2 µg orexin), 3 (LE and 1 µg orexin) and 4 (LE and 1 µg orexin) and before, during and after infusions of ghrelin. NE: Normal energy; LE: Low energy. Treatments with different letter(s) are different at $p<0.01$

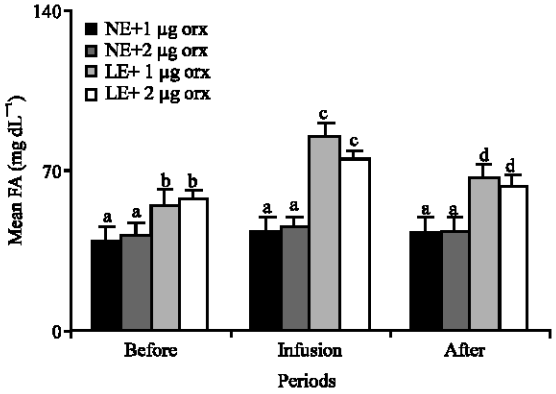

Fig. 7: Mean plasma concentrations of fatty acid of the animals in the different groups of 1 (NE and 1 µg orexin), 2 (NE and 2 µg orexin), 3 (LE and 1 µg orexin) and 4 (LE and 1 µg orexin) and before, during and after infusions of ghrelin. NE: Normal energy; LE: Low energy. Treatments with different letter(s) are different at $p<0.01$

LE. Mean plasma concentrations of fatty acid of the animals in the group 3 (55 mg dL^{-1}) and 4 (57mg dL^{-1}) fed LE were significantly ($p<0.01$) higher than the mean plasma concentrations of fatty acid of those animals in groups 1 (40 mg dL^{-1}) and 2 (42 mg dL^{-1}) fed NE (Fig. 7). Infusions of 1 and 2 µg orexin significantly ($p<0.01$) increased the fatty acid levels among those animals fed LE (Fig. 7).

Urea: Orexin significantly ($p<0.01$) increased the mean plasma concentrations of the urea of the animals in all groups. Mean plasma concentrations of urea of the animals in group 3 and 4 fed LE were significantly ($p<0.01$) higher than the mean plasma concentrations of urea of those NE fed animals in groups 1 and 2 (Fig. 8).

Body weight: Low energy dietary intake for 20 days significantly ($p<0.01$) decreased the mean body weight of the animals from 47 to 35 kg. This was similar to our previous finding reported that negative energy balance decrease body weight in the ewes (Towhidi *et al*., 2007).

DISCUSSION

T3 and T4: Present study is the first to report the effect of orexin into the third ventricle on thyroid hormones in the ruminants. The results of the effect of orexin on mean plasma T3 and T4 levels of the goats fed LE is different from the previous finding of (Jones *et al*., 2001; Mitsuma *et al*., 1999; Russell *et al*., 2001, 2002) that reported the central effect of orexin decreased the plasma level of Thyroid Stimulating Hormones (TSH) in nonruminants such as rat, but there was no data on the plasma level of T3 and T4 in that study. It is well established that decrease of plasma TSH level is accompanied by increase of plasma T3 and T4 in NE fed nonruminant (Felig and Frohman, 2001; Reasner and Ralbert, 2002). Present results indicate that the NE fed goats as a ruminant response differently to orexin as that of in nonruminant. Only when the ruminant animals are in long term fasting period, are sensitive to the effect of orexin on plasma T3 and T4 levels. The Hypothalamus

Pituitary Thyroid (HPT) axis plays important role in the regulation of energy homeostasis (De Jesus *et al*., 2001; Lanni *et al*., 2001) via the effects of thyroid hormone to increase oxygen consumption and heat generation (De Jesus *et al*., 2001; Lanni *et al*., 2001). Thus, inhibition of the HPT axis during fasting would appear to be an important adaptive mechanism to conserve energy stores (Rondeel *et al*., 1992; Van Haasteren *et al*., 1995; Legradi *et al*., 1997). The state of central hypothyroidism induced by fasting is orchestrated by changes of circulating levels of orexin, which rise with fasting and is restored to normal levels by refeeding (Rondeel *et al*., 1992). Thus, if orexin is administered exogenously to fasting animals, the more decrease in circulating levels of thyroid hormones can be observed (Legradi *et al*., 1998).

GH: Present study is also the first to report the effect of orexin into the third ventricle on GH in the ruminants fed LE. Our result about the effect of orexin on GH in the goats fed LE is different from other studies indicated that orexin decreased GH secretion and enhances the GH response to somatostatin in NE fed nonruminant (Hagan *et al*., 1999; Lopez *et al*., 2004; Overeem *et al*., 2003; Willie *et al*., 2002). Furthermore, conflicting evidence exist *in vitro* about the direct effect of orexin on GH, with an no influence on GH secretion observed in rat (Samson and Taylor, 2001). This may be due to decreased plasma level of insulin and glucose (Holl *et al*., 1999) in the goats fed LE.

Insulin: Our data are different from the studies in nonruminants that indicated orexin has no effect or increase the plasma level of insulin (Meghan and Samson, 2003; Nowak *et al*., 2005). In those studies, the effect of orexin was not on the long term fasting subject. The mechanism of inhibitory effect of orexin on insulin release most likely maybe like galanin that occurs through the inhibition of adenylate cyclase, involving a petussis-toxin-sensitive inhibitory GTP-binding regulatory protein and the activity of protein kinase C and cyclic AMP (Amiranoff *et al*., 1988; Lindskog and Ahren, 1991).

Glucagon: Present results are different from the previous studies that reported orexin inhibit glucagon secretions *in vitro* (Göncz *et al*., 2008; Quedraogo *et al*., 2003). This may be due to the plasma glucose concentrations (Manabe *et al*., 2003). The above study were conducted to determine the effect of orexin on glucagon *in vitro*. In present study, decreased plasma level of glucose caused by lower energy intake (Marsoobian *et al*., 1995) and orexin infusions may be the reason for increase level of glucagon and decrease level of insulin.

Glucose, fatty acid and urea: It is well established that low energy content in diet decreases mean plasma concentrations of glucose in most mammals (Marsoobian *et al*., 1995) as we observed in the goats fed LE. Also, there is a negative correlation between orexin infusion and mean plasma level of glucose in the fasted ruminants whereas in other study is reported that there is a positive correlation between these two parameters in nonfasted nonruminant (Leibowitz *et al*., 1998).

Present result bout the effect of orexin on urea in the LE fed goats is similar to other study done in the nonruminant that indicated low energy diet increased plasma urea level (Khazali, 1992).

When energy intake is inadequate, proteins can serve as an energy source and plasma urea level is considered as an endproduct of protein catabolism (Ruiz *et al*., 1971).

Implication: The results of our studies indicated that the third ventricle infusion of orexin may increase the plasma levels of GH, glucagons, fatty acid and urea and decrease the plasma levels of T3, T4, insulin and glucose in the goats with severe body loss. The effect of orexin infusion into third ventricle on metabolic parameters is different from the effect of orexin injections in peripheral circulation. Also, different metabolic system of ruminant and nonruminant animals offer different changes of metabolic status under orexin effect.

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