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Decreased serum osmolality promotes ductus arteriosus constriction

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Aims At birth, dynamic changes occur in serum components and haemodynamics, such as closure of the ductus arteriosus (DA). A previous study demonstrated that, in full-term human neonates, serum osmolality decreased transiently after birth, but recovered over the next few days. However, the significance of this transient decrease in osmolality has never been addressed. The objective of the present study was to examine the role of changes in serum osmolality after birth in DA closure.

Methods and results We found that rats exhibited a similar transient hypoosmolality after birth. Hypotonic stimulation induced constriction of DA rings and increased Ca^{2+} transient in DA smooth muscle cells, but not in the aorta. The hypoosmotic sensor transient receptor potential melastatin 3 (TRPM3) was highly expressed in the rat DA, and TRPM3 silencing abolished the Ca^{2+} response to hypoosmolality. Pregnenolone sulfate stimulation of TRPM3 induced rat DA constriction *ex vivo* and *in vivo*. Furthermore, *hyper*tonic fluid injection impaired rat DA closure. In humans, neonatal serum hypoosmolality was observed in relatively mature preterm infants (≥28 weeks). In extremely preterm infants (<28 weeks), however, this hypoosmolality was absent. Instead, a rapid increase in osmolality occurred thereafter. Such an increase was greater, in particular, among patent DA (PDA) patients.

Conclusions A transient decrease in serum osmolality may promote DA closure during the first few days of life. Adjusting serum osmolality to proper levels might help to prevent the onset of PDA, improving the therapeutic outcome in extremely preterm infants.

Keywords Patent ductus arteriosus • Paediatrics • Biology • Developmental • Osmolality • TRP channel

1. Introduction

At birth, the separation of the foetus from its maternal environment constitutes a dramatic change for the infant, as it moves from an aquatic to an atmospheric environment. Infants must start breathing with their lungs immediately and are suddenly exposed to conditions of high oxygen tension. It appears that certain specific physiological processes take place in reaction to these environmental stresses to modify the cardiovascular system and thus rapidly adapt the infant to the postnatal environment. A prime example is the closure of the ductus arteriosus (DA), which is an arterial shunt vessel connecting the pulmonary artery with the aorta.[1]

In the foetus, the DA diverts ventricular output away from the high-resistance pulmonary vascular bed into systemic circulation. Although its patency is essential for foetal circulation, once pulmonary circulation is established, the DA must be closed. Persistent patent DA (PDA) after

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birth often causes serious problems in very premature infants, depending on the degree of left-to-right shunting. It has been reported that more than 50% of infants with birth weights <1000 g receive pharmacological therapy consisting of cyclooxygenase (COX) inhibitors or surgical ligation of the DA to prevent respiratory distress, heart failure, intraventricular haemorrhage/brain injury, bronchopulmonary dysplasia, or necrotizing enterocolitis.[2–5] Because the current pharmacological and surgical therapies for treating PDA are not always ideal,[6–8] it is highly desirable to find a relatively simple and non-invasive strategy that would prevent PDA from occurring.

It is well known that increasing oxygen tension in the arteries promotes DA constriction by inhibiting voltage-gated potassium channels[9,10] and activating voltage-gated calcium channels.[11–13] Simultaneous withdrawal of placental prostaglandin E_2 (PGE_2) and promotion of PGE_2 catabolism in the lungs also contribute to DA closure.[14–16] Thus, changes in serum composition, as induced by the initiation of respiration, significantly affect closure of the DA. The onset of respiration is also known to facilitate alveolar fluid absorption in the lungs,[17–19] which may also lead to changes in serum osmolality. In this regard, it was reported convincingly by Feldman and Drummond[20] that full-term infants exhibit low serum osmolality for the very first few days of life, and that their level of serum osmolality reaches adult levels by the fifth day of life. The significance of this transient decrease in serum osmolality has been largely overlooked in infants, including premature infants, who frequently suffer from PDA.

Under physiological conditions, body fluid osmolality is tightly regulated to near 300 mOsm/kg through the intake or excretion of water and salt.[21] This homeostatic osmoregulation is vital, and hypotonicity is sensed, at least in part, by transient receptor potential (TRP) channels, such as TRP vanilloid 4 (TRPV4)[22] or TRP melastatin 3 (TRPM3),[23,24] which act as Ca^{2+} channels. Because changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) are related to DA constriction,[11–13] we hypothesized that the transient decrease in serum osmolality may contribute to DA closure, potentially via TRP channels.

In this study, we demonstrated a similar transient decrease in serum osmolality at birth in rat neonates, as previously reported in human neonates by Feldman *et al.*[20] Furthermore, we found that hypoosmolality contributed to the constriction of the DA through the activation of TRPM3 in rats. We also demonstrated that this transient decrease is observed in relatively mature human preterm infants, but is absent in extremely preterm infants. Extremely preterm infants with PDA, in particular, exhibited early elevation of serum osmolality. We thus suggest that serum osmolality plays an important role in regulating DA constriction, and that adjusting serum osmolality to proper levels might contribute to DA closure among extremely preterm infants.

2. Methods

Detailed information in the Methods section used in this study can be found in Supplementary Material online.

2.1 Reagents

Anti-TRPM3 antibody was purchased from Novus Biologicals (Littleton, CO, USA). Pregnenolone sulfate, a TRPM3 channel stimulator, was purchased from Sigma-Aldrich (St Louis, MO, USA). Isosakuranetin, a TRPM3 channel inhibitor, was purchased from Extrasynthese (Genay, France). Nicardipine hydrochloride was purchased from Wako (Osaka, Japan). Indomethacin was purchased from Calbiochem (San Diego, CA, USA). KB-R7943, an NCX inhibitor, was purchased from Calbiochem.

2.2 Animal studies

Wistar rat foetuses and neonates were obtained from timed-pregnant mothers purchased from Japan SLC, Inc. (Shizuoka, Japan). The experiments were approved by the Yokohama City University Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals (reference number: F-A-13-006).

2.3 Human studies

Protocols for using blood samples and human tissues were approved by the Research Ethics Committee in Yokohama City University Hospital and Kanagawa Children's Medical Center (reference numbers: B100107034 and D13021010) and conformed to the principles outlined in the Declaration of Helsinki.

2.4 Primary culture of rat DA and aorta smooth muscle cells

Immediately after euthanasia of the mother rat using 200 mg/kg of pentobarbital, the foetuses were obtained by the caesarean section. After decapitation of the foetuses, vascular smooth muscle cells (SMCs) in primary culture were obtained from the DAs (DASMCs) and the aortas (ASMCs) of rat foetuses at the 21st day of gestation (e21) as previously described.[12,25] A single line of SMCs were obtained from about 10 rats. More than four lines of SMCs were used for each experiment. The subconfluent cells of three to six passages were used in the experiments.

2.5 Serum osmolality measurement

Serum osmolalities during the perinatal period in rats and humans were analysed by a freezing-point depression osmometer (model 210, Fiske Associates, Norwood, MA, USA). Blood samples from rat foetuses at e21 and infant rats at Day 0 (1 or 2 h after birth), Day 1, Day 2, and Day 7 were obtained by heart puncture after rats were anaesthetized with isoflurane. To obtain e21 rat foetuses and Day 0 rat infants by the caesarean section, the maternal rats at e21 were euthanized with 200 mg/kg of pentobarbital. The rat neonates over Day 1 were normally delivered and raised by their mothers until the day of measurement.

Human infants were enrolled in the study after parental written informed consent was obtained. Sixty-two total preterm infants who were admitted to the neonatal intensive care unit (NICU) of Yokohama City University Hospital at ≥24 and <36 weeks of gestation were recruited prospectively between October 2010 and March 2013. Exclusion criteria were major congenital anomalies, congenital heart diseases, and death in the first week of life. The patients were divided into two groups: those with ≥28 weeks of gestation and those with <28 weeks of gestation. The <28-week group was subdivided into PDA and non-PDA groups. In the ≥28-week group, there was only one patient with PDA. In this study, PDA was defined as the necessity of indomethacin therapy within the first week of life. Indomethacin is not prophylactically administrated at this institution. The patients were followed up until 7 days after birth.

Clinical backgrounds were recorded and are summarized in *Table 1*. Extremely preterm infants with PDA were treated with intravenous injection of indomethacin between Day 1 and Day 5 (2.1 ± 1.4 days). None of the infants received indomethacin before the osmolality measurement of Day 1, and 11 of 15 cases (73.3%) received this therapy on Day 1 or Day 2. Respiratory distress syndrome (RDS) was defined as any situation in which surfactant was administered for therapy. Cord blood (CB) samples were obtained at the time of delivery and samples from neonates were collected on admission to the NICU (1 h after birth) and on Days 1 (9–29 h after birth) and 2 (33–53 h after birth) after birth. All the samples used to test serum osmolality were centrifuged at 845 *g* for 5 min and the serum samples were kept frozen at −30°C until analysis.

Table 1 Characteristics of the preterm infant groups

Variables	A	B	C	P-value			
	28–35 weeks (n = 35)	24–27 weeks without PDA (n = 12)	24–27 weeks with PDA (n = 15)	A vs. B vs. C	A vs. B	A vs. C	B vs. C
Gestational age (weeks)	32.56 ± 2.20	26.6 ± 1.06	26.1 ± 0.95	<0.001‡	<0.001‡	<0.001‡	0.365
Birth weight (g)	1617.1 ± 512.5	823.0 ± 244.7	782.9 ± 201.6	<0.001‡	<0.001‡	<0.001‡	0.733
Male, n (%)	20 (57%)	5 (42%)	9 (60%)	0.584			
RDS, n (%)	8 (23%)	8 (67%)	10 (67%)	0.003†	0.012*	0.009†	1.000
Maximum FiO_2 in 12 h	0.32 ± 0.15	0.53 ± 0.25	0.48 ± 0.21	<0.001‡	0.001†	0.003†	0.641
SFD, n (%)	10 (29%)	5 (42%)	3 (20%)	0.430			
Apgar score at 1 min	6.9 ± 2.1	5.4 ± 2.4	5.0 ± 1.5	0.002†	0.027*	<0.001‡	0.603
Apgar score at 5 min	8.3 ± 1.5	7.3 ± 1.7	7.4 ± 1.6	0.019*	0.094	0.016*	0.980
Infection, n (%)	1 (3%)	2 (17%)	2 (13%)	0.244			
Antenatal steroids, n (%)	15 (43%)	11 (92%)	14 (93%)	<0.001‡	0.006†	0.001†	1.000
Furosemide, n (%)	0 (0%)	0 (0%)	2 (14%)	0.172			

RDS: respiratory distress syndrome; SFD: small for date.
*$P < 0.05$.
†$P < 0.01$
‡$P < 0.001$ statistically significant.

2.6 Osmolyte measurement

Serum Na^+, K^+, glucose, and urea nitrogen (BUN) were measured using an i-STAT blood analysis system (Abbott Laboratories, Princeton, NJ, USA).

2.7 RNA isolation and quantitative reverse transcription–polymerase chain reaction

Pooled tissues of the DA and the aorta were obtained from rat e19 foetuses ($n > 40$), e21 foetuses ($n > 40$), and neonates on the day of birth (Day 0, $n > 40$). Before tissue collection, rat foetuses were decapitated and neonates were anaesthetized with isoflurane. After the total RNA was isolated, reverse transcription–polymerase chain reaction was performed using a PrimeScript RT reagent kit (TaKaRa Bio, Tokyo, Japan) and SYBR Green (Applied Biosystems, Foster City, CA, USA). The sequences of the primers for the TRP isoforms are described in Supplementary material online, *Table S1*.

2.8 Immunohistological staining

DA tissues from rat Day 0 neonates and DA tissues from human neonates obtained during cardiac surgery were stained for TRPM3.

2.9 Isometric tension of the DA vascular rings

Isometric tension of the vascular rings of the DA ($n = 8$) and the aorta ($n = 7$) from rat e21 foetuses was measured as previously described.[13,26] The DA and aortic rings were independently isolated after decapitation from eight and seven rats, respectively. After a vasoconstriction plateau had been attained, osmolality of the bath fluid was reduced step by step from 300 to 220 mOsm/kg. Vasoconstriction at 250 mOsm/kg was also observed over 30 min in other DA rings ($n = 5$). Vasoconstriction induced by pregnenolone sulfate, a TRPM3 channel stimulator, was measured in the DA rings. The vasoconstrictions induced by hypoosmolality (250 mOsm/kg, $n = 4$) or pregnenolone sulfate (200 μmol/L, $n = 4$) were measured under pre-treatment of the TRPM3 inhibitor isosakuranetin[27,28] (25 μmol/L). Hyperosmotic relaxation of the DA ring was evaluated in indomethacin-induced DA constriction. The degree of vascular constriction was expressed as a percentage of the constriction induced by 120 mmol/L of KCl.

2.10 Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in SMCs

DASMCs or ASMCs obtained from more than 40 foetuses were placed in 96-well microplates at 1×10^4 cells/well. After 2 days of incubation, SMCs were loaded with 5 μmol/L of fura-2/AM (Dojindo, Kumamoto, Japan) in Tyrode solution for 20 min at 37°C. Hypotonic stimulation was induced by adding Tyrode solution containing no NaCl into each well, which induced an osmolality reduction by changing the NaCl concentration. The data were obtained from four independent experiments assayed in triplicate using four lines of SMCs.

2.11 RNA interference

Small interfering RNA (siRNA) of TRPM3 and control siRNA were purchased from Thermo Fisher Scientific (Waltham, MA, USA). According to the manufacturer's instructions, cultured DASMCs were transfected with siRNA targeted for TRPM3 using Lipofectamine RNAiMAX (Invitrogen).[26] We examined the mRNA expressions of all TRPM isoforms, and found that TRPM3, TRPM4, and TRPM7 were abundantly expressed in the rat DA, and expressions of the other isoforms were faint (data not shown). TRPC1 has been reported to contribute to DA contraction,[29] and TRPV4 is known to be activated with hypoosmolality.[22] Therefore, we examined the off-target effect of TRPM3-targeted siRNA on the abovementioned TRP channels. Efficacy of TRPM3 silencing and off-target effects on other TRP isoforms were analysed using the data from four lines of SMCs.

2.12 Rapid whole-body freezing method

A whole-body freezing method was used to examine the *in situ* morphologies and inner diameters of foetal and neonatal rat DA specimens as previously described.[26] Briefly, after the maternal rats were anaesthetized with isoflurane, *in utero* foetuses at e21 were injected intraperitoneally with pregnenolone sulfate (100 mg/kg of body weight) or indomethacin (2 mg/kg of body weight). The adequacy of anaesthesia was monitored by assessment of skeletal muscle tone, respiration rate, and response to tail pinch. After 1 h, they were delivered by the caesarean section and immediately frozen in liquid nitrogen before breathing. Other rat neonates delivered at e21 by the caesarean section were injected intraperitoneally with NaCl solution (0.15–1.5 mol/L) at birth to modulate serum osmolality. After 30 min, rat neonates were anaesthetized with isoflurane and frozen in liquid nitrogen. After caesarean section, the maternal rats were euthanized by 200 mg/kg of pentobarbital. The frozen specimens were sectioned under a microscope, and the inner diameters of the DA, the pulmonary artery (PA), and the aorta were measured. DA/PA ratios were used to evaluate *in vivo* DA constriction.

2.13 Statistical analysis

All values are shown as the mean ± SEM of more than three independent experiments, except the characteristics of the patients, which are expressed as the mean ± SD. Detailed statistical analysis is shown in Supplementary material online. A value of $P < 0.05$ was considered significant.

3. Results

3.1 Hypotonic stimulation contracted the rat DA

As reported previously in human neonates,[20] we found that serum osmolality was also transiently decreased in rat neonates. In full-term rat neonates, it was decreased by >20 mOsm/kg within 1 h after birth (*Figure 1A*) and reached normal adult levels over the next several days. We then examined the potential role of this hypoosmolality in the

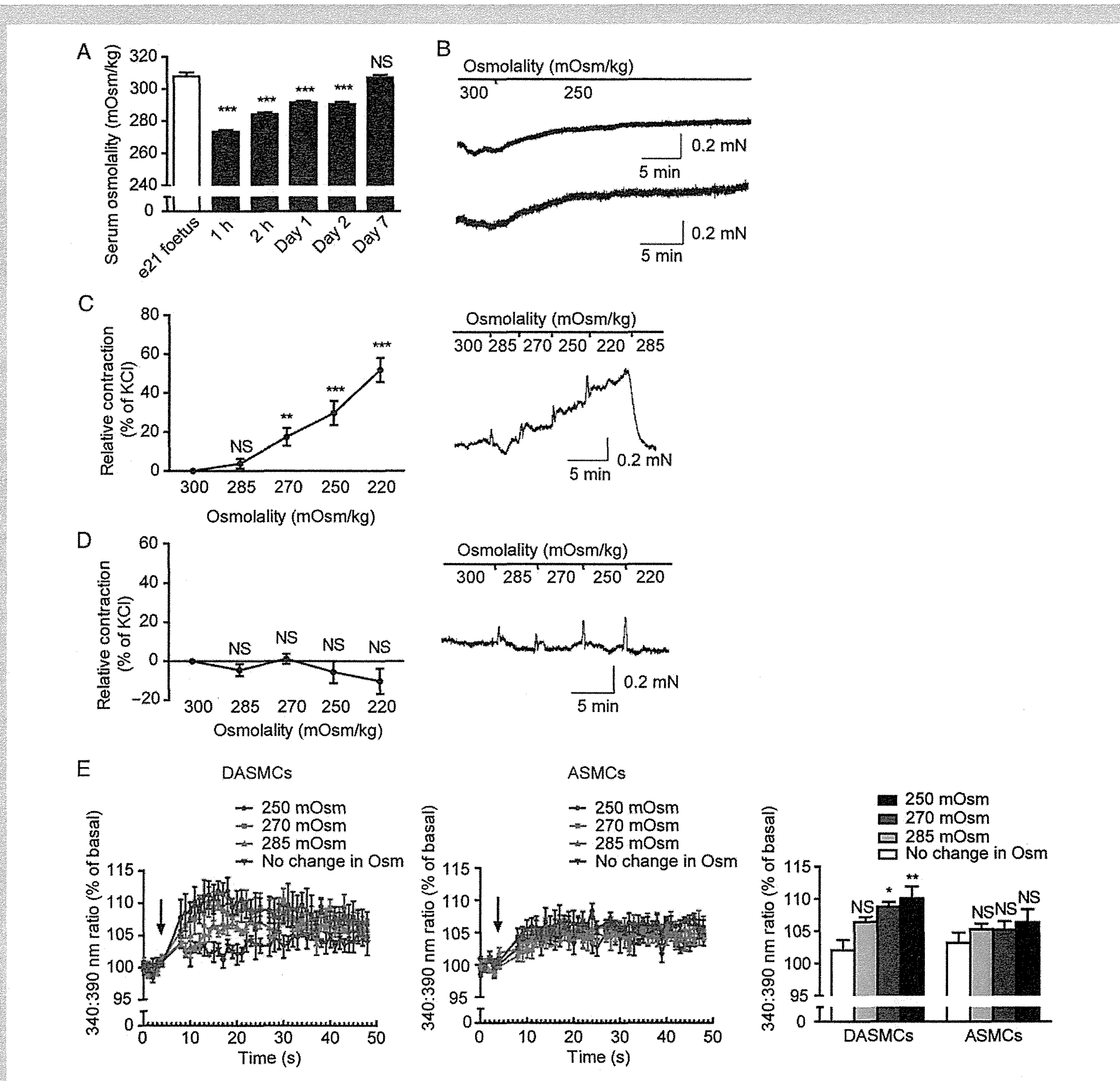

Figure 1 Effect of hypotonic stimulation on DA contraction and $[Ca^{2+}]_i$ transients in rats. (*A*) The time course of serum osmolalities in full-term rat neonates shows the transient decrease in serum osmolality after birth. $n = 4–8$, $***P < 0.001$ vs. e21 foetus. NS: not significant. (*B*) Representative myograph traces of tension in the rat DA rings stimulated with hypoosmolality (250 mOsm/kg). (*C* and *D*) Effects of hypotonic stimulation on the DA (*C*) and aorta (*D*), with representative myographs. Contraction of vascular rings from full-term e21 rat foetus was measured using a wire myograph. $n = 7-8$, $**P < 0.01$; $***P < 0.001$ vs. 300 mOsm/kg. NS: not significant. (*E*) $[Ca^{2+}]_i$ transients were measured using fura-2 in DASMCs (left) and ASMCs (middle). Cells were incubated at 300 mOsm/kg, followed by hypotonic stimulation as indicated by arrows. Quantification at 15 s after hypotonic stimulation shows that $[Ca^{2+}]_i$ transients were specific to the DA (right). $n = 4$, $*P < 0.05$; $**P < 0.01$ vs. no change in osmolality. NS: not significant; mOsm: mOsm/kg.

DA, using isolated rat DA rings and a wire myograph. Hypoosmolality was generated by modulating the Na^+ concentration, a major component in serum osmolality. We found that rat DA rings contracted at 250 mOsm hypotonic stimulation (*Figure 1B*), and that the contraction was in a hypoosmolality-dependent manner (down to 220 mOsm) (*Figure 1C*, left). This contraction was reversible when osmolality was restored to 285 mOsm (*Figure 1C*, right). In contrast, hypoosmolality-induced contraction was absent in the aortic tissue rings (*Figure 1D*). It is unlikely that the Na/Ca exchanger (NCX) plays a role in this hypoosmolality-induced regulation, since inhibition of NCX by KB-R7943 did not alter hypoosmolality-induced DA contraction (see Supplementary material online, *Figure S1*).

To explore the molecular mechanisms for this DA contraction, we measured $[Ca^{2+}]_i$, which most likely regulates DA constriction, using fura-2 in DASMCs and ASMCs. When these tissues were exposed to a hypotonic solution, $[Ca^{2+}]_i$ was increased in an osmolality-dependent manner in DASMCs (*Figure 1E*, left), but not in ASMCs (*Figure 1E*, middle). Thus, osmotic sensor(s) appear to play a role only in DASMCs (*Figure 1E*, right). These data suggest that serum osmolality is physiologically decreased after birth, and that the hypoosmolality induces a postnatal contraction in the DA by regulating $[Ca^{2+}]_i$.

3.2 Hypoosmotic sensing is mediated by TRPM3 in rat DASMCs

TRP channels are known to act as osmotic sensor molecules. In particular, it has been reported that TRPV4 and TRPM3 are activated by hypoosmolality.[22–24] We found that mRNA expression of TRPM3 was greater in the rat DA than in the aorta during development, i.e. preterm (e19) and full-term (e21) foetuses as well as normal newborn neonates (d0) (*Figure 2A*). In contrast, TRPV4 mRNA expression was similar in both the DA and the aorta (*Figure 2B*). The protein expression of TRPM3 was most abundant in the medial layer of the rat DA (*Figure 2C*) and in the human DA (see Supplementary material online, *Figure S2*). We also examined the role of TRPM3 in hypoosmolality-induced elevation of $[Ca^{2+}]_i$. Hypotonic stimulation induced changes in $[Ca^{2+}]_i$ (*Figure 1E*), but such changes disappeared when TRPM3 was silenced by siRNA (*Figure 3A–C*). The TRPM3-targeted siRNA did not decrease some other TRP channels, i.e. TRPC1, TRPV4, TRPM4, and TRPM7 (see Supplementary material online, *Figure S3*). In addition, when DASMCs were preincubated with the TRPM3 inhibitor isosakuranetin, hypotonic stimulation-induced $[Ca^{2+}]_i$ elevation was attenuated (*Figure 3D*). The voltage-gated Ca^{2+} channel is activated by TRP channel-mediated Ca^{2+} influx. We therefore then examined the involvement of a voltage-gated Ca^{2+} channel, and found that the voltage-gated Ca^{2+} channel blocker nicardipine did not suppress the hypoosmolality-induced $[Ca^{2+}]_i$ elevation (*Figure 3E*). Thus, expression of TRPM3 may be responsible for osmolality-induced contraction of the DA.

3.3 Pharmacological stimulation of TRPM3 caused contraction of the rat DA

Similarly, pregnenolone sulfate, a TRPM3 stimulator,[30] increased the rat DA ring tension *in vitro* in a dose-dependent manner (*Figure 4A*). We also injected pregnenolone sulfate into rat foetuses intraperitoneally *in utero* and found that DA closure was moderately but significantly facilitated (*Figure 4B*), although this contraction was less than that caused by indomethacin, a COX inhibitor and thus a potent DA contracting drug. Furthermore, inhibition of TRPM3 using isosakuranetin attenuated the DA ring contraction induced by hypoosmotic stimulation (*Figure 4C*) or pregnenolone sulfate stimulation (*Figure 4D*). Taken together, these findings suggest that changes in osmolality regulate Ca^{2+} transients in the DA via TRPM3, resulting in DA contraction, at least, in rats.

3.4 Serum hyperosmolality suppressed DA closure

Based on the above findings, we assumed that serum *hyper*osmolality would, in contrast, inhibit DA closure. We injected the same volume of various concentrations of NaCl into rat neonates to increase their serum osmolality. Injecting 0.15 mol/L of NaCl resulted in 285.8 mOsm/kg of serum osmolality and did not alter DA closure (*Figure 5A–B*). In contrast, injecting higher concentrations of NaCl (0.6–1.5 mol/L) increased serum osmolality and inhibited DA closure in a serum osmolality-dependent manner (*Figure 5A–B*). We then examined the involvement of the inhibition of TRPM3 in *hyper*osmolality-induced DA dilation. We found that *hyper*osmolality dilated indomethacin-induced DA ring contraction; however, this was not affected by the inhibition of TRPM3 through the use of isosakuranetin (*Figure 5C*). These findings suggest that an acute increase in serum osmolality could inhibit DA closure, although this does not appear to involve TRPM3.

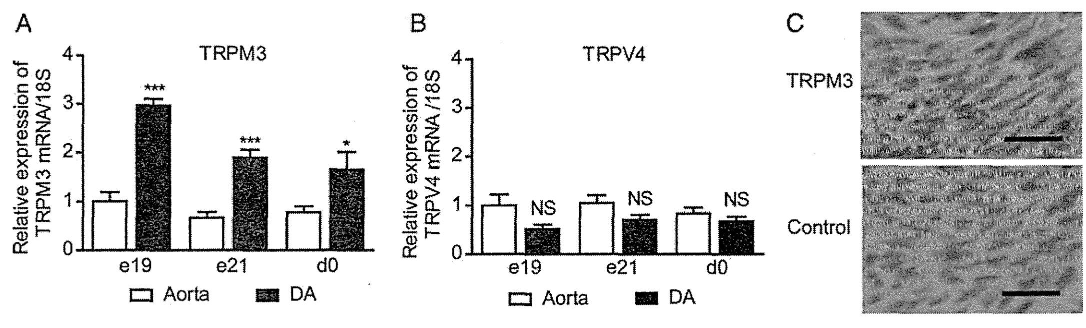

Figure 2 Expression of TRPM3 in the rat DA. (*A* and *B*) Developmental changes in the expression of TRPM3 and TRPV4 mRNA in the rat DA and aorta. $n = 4–5$, $*P < 0.05$; $***P < 0.001$ vs. the aorta. NS: not significant. (*C*) Brown colour indicates TRPM3 protein expressed in the medial layer of rat DA. Negative control (lower panel) was obtained by omission of a primary antibody. Scale bars: 20 μm.

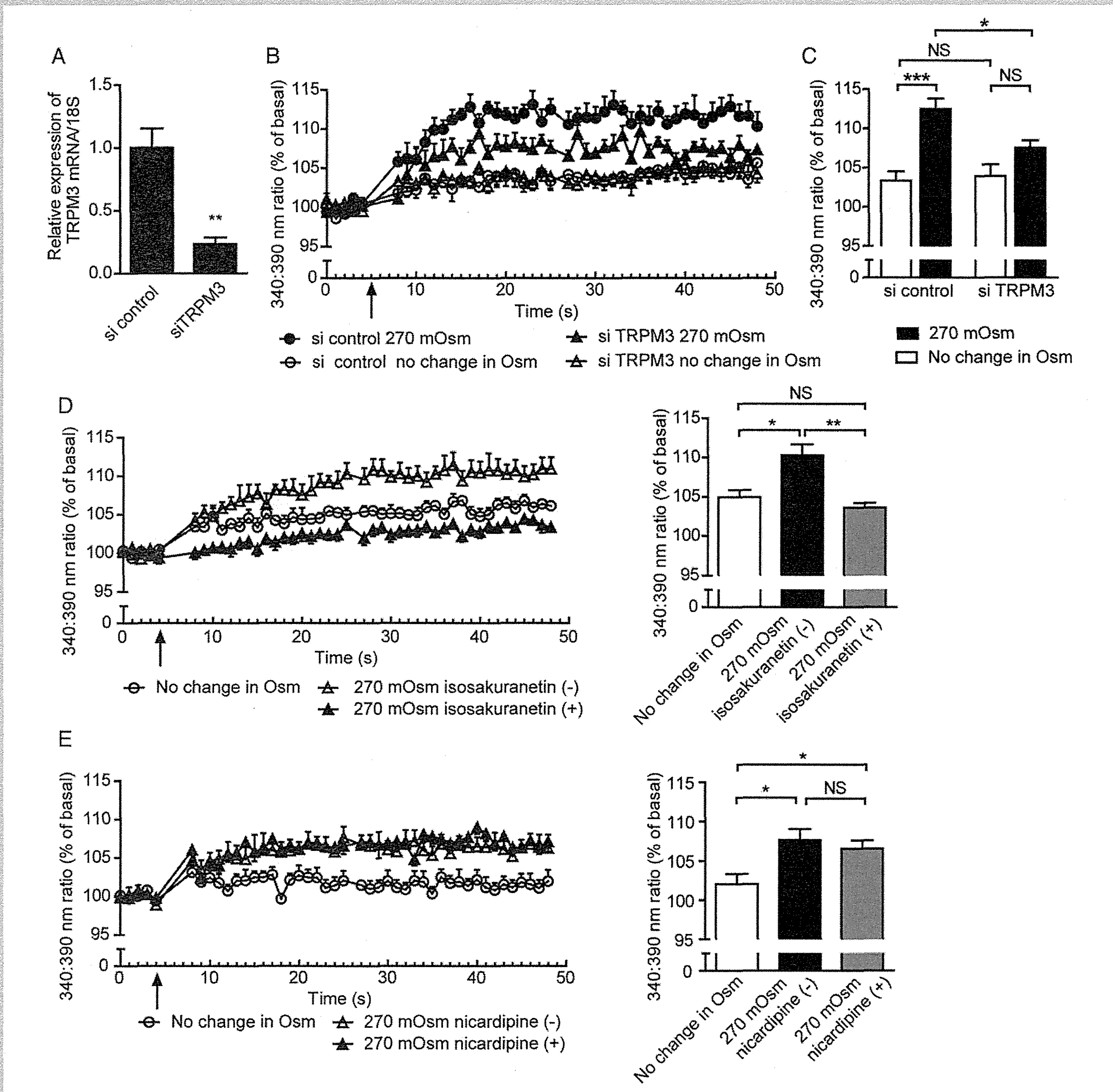

Figure 3 Hypoosmolality-induced elevation of $[Ca^{2+}]_i$ in DASMCs. (*A*) TRPM3 mRNA expression was decreased by TRPM3-targeted siRNA (si TRPM3). $n = 6$, $^{**}P < 0.01$ vs. control siRNA (si control). (*B*) $[Ca^{2+}]_i$ transients were measured using fura-2 in DASMCs treated with TRPM3-targeted siRNA or control siRNA. Cells were incubated at 300 mOsm/kg, followed by hypotonic stimulation (270 mOsm/kg) as indicated by the arrow. $n = 4$. (*C*) At 15 s after hypotonic stimulation, it can be seen that si TRPM3 eliminated the hypotonically induced $[Ca^{2+}]_i$ transients. $n = 4$, $^{*}P < 0.05$; $^{***}P < 0.001$; NS: not significant. (*D*) Preincubation of the TRPM3 inhibitor isosakuranetin (25 μmol/L) eliminated the hyposmotically induced $[Ca^{2+}]_i$ transients. $n = 5$, $^{*}P < 0.05$; $^{**}P < 0.01$; NS: not significant. (*E*) Preincubation of nicardipin (10 μmol/L) did not alter the hypotonically induced $[Ca^{2+}]_i$ transients. $n = 5$, $^{*}P < 0.05$; NS: not significant. mOsm: mOsm/kg.

3.5 Postnatal changes of serum osmolality in human preterm infants

Our data in rats demonstrated that *hypo*osmolality promoted DA constriction and that *hyper*osmolality inhibited DA closure. Since PDA develops in preterm infants in humans, we examined the relationship between PDA and serum osmolality. We measured serum osmolality of preterm infants in three groups: (i) infants delivered at 28–35 weeks of gestation (28–35-WG, moderate-preterm, and late-preterm infants, $n = 35$), (ii) infants delivered at 24–27 weeks of gestation with PDA (24–27-WG PDA+, extremely preterm infants, $n = 15$), and (iii) infants born at 24–27 of gestation without PDA (24–27-WG PDA−, extremely preterm infants, $n = 12$) (*Table 1*). Serum osmolality was determined with CB (equivalent to foetal blood at the time of birth), infant blood at Day 0 (1 h after birth), Day 1, and Day 2.

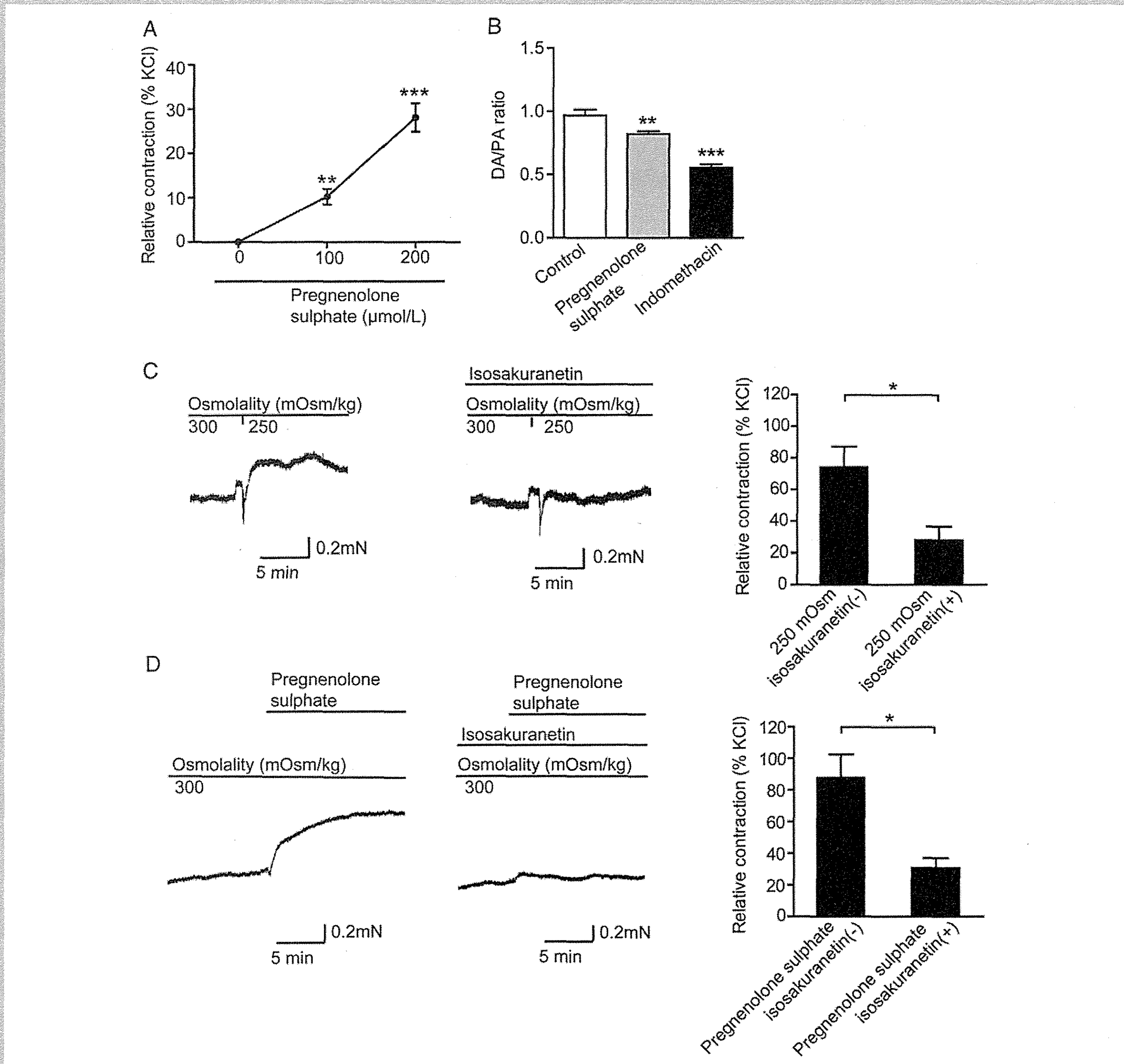

Figure 4 Effect of pregnenolone sulfate on rat DA contraction. (*A*) The TRPM3 activator pregnenolone sulfate constricted the rat e21 DA. Vascular tension was measured using a wire myograph. $n = 6$, $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control. (*B*) Intraperitoneal administration of pregnenolone sulfate (100 mg/kg body weight) or indomethacin (2 mg/kg body weight) constricted e21 foetal DA *in vivo*. Diameters of the DA and the main trunk of the pulmonary artery were measured by the whole-body freezing method. $n = 4–9$, $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control. (*C* and *D*) Isosakuranetin (25 μmol/L) attenuated the hypoosmotically- and pregnenolone sulfate (200 μmol/L)-induced DA contraction, respectively. Vascular tension was measured using a wire myograph. $n = 4–6$, $^{*}P < 0.05$ (*C*), $n = 4–5$, $^{*}P < 0.05$ (*D*). mOsm: mOsm/kg.

First we analysed all the groups of infants and found that serum osmolality significantly decreased at birth and gradually recovered to levels of CB (*Figure 6A*). In accordance with these data, in 28–35-WG infants, serum osmolality was decreased significantly after birth (CB vs. Day 0; 284.2 ± 1.6 vs. 275.1 ± 1.5, $P < 0.001$) and recovered to levels of CB at Day 2 (284.2 ± 1.6 vs. 284.9 ± 1.9, NS; *Figure 6B*). This pattern was consistent with data on full-term human[20] and rat neonates. In both 24–27-WG groups, however, serum osmolality was scarcely decreased at Day 0, but was significantly elevated thereafter (Days 1 and 2; *Figure 6B*). Importantly, this elevation was significantly greater in the 24–27-WG PDA+ group than in the 24–27-WG PDA− group or the 28–35-WG group (*Figure 6C*). Such dynamic differences were observed only between Day 0 and Day 1 among the three groups (*Figure 6D–E*). Thus, relatively mature infants (28–35-WG) exhibited a transient decrease and gradual recovery in serum osmolality after birth. This decrease did not occur in immature infants (24–27-WG) and rather steeply increased between Day 0 and Day 1, and this increase was much greater in infants with PDA.

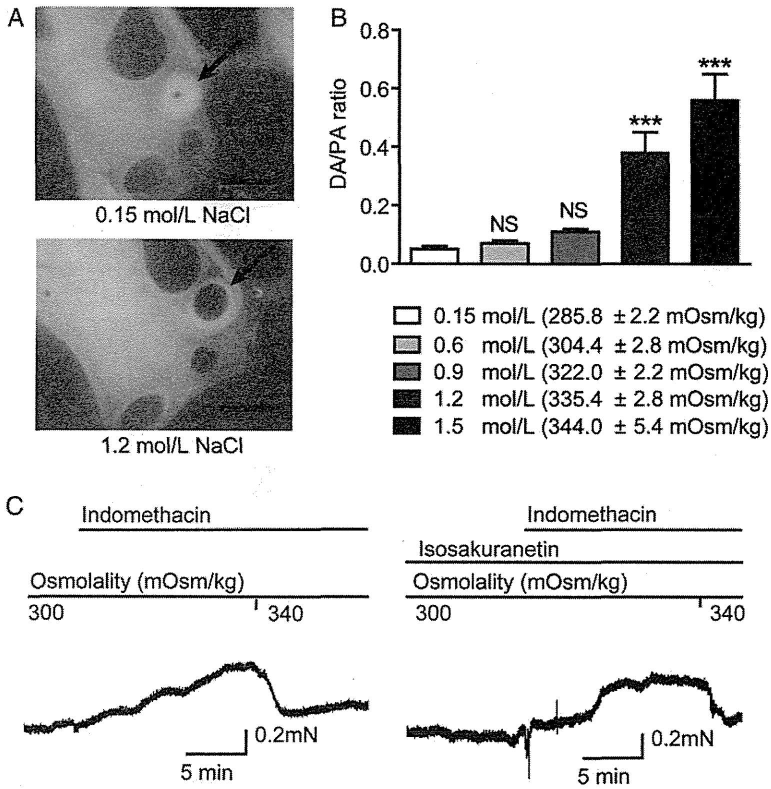

Figure 5 Inhibition of DA closure by hypertonic stimulation in rat neonates. (*A*) Representative images of rat DAs (arrows) 30 min after hypertonic stimulation with 100 μL of 0.15 mol/L NaCl (normal saline) or 1.2 mol/L NaCl. Hypertonic stimulation was induced by an intraperitoneal injection of NaCl at birth, and its effect was measured 30 min after birth using the whole-body freezing method. Scale bars: 500 μm. (*B*) Hypertonic stimulation inhibited the DA closure *in vivo*. $n = 4-8$, ***$P < 0.001$ vs. 0.15 mol/L. NS: not significant. (*C*) Hypertonic stimulation dilated the rat DA rings under stimulation of indomethacin ($1 \times 10^{-5.5}$ mol/L), which was not modulated by isosakuranetin (25 μmol/L). Vascular tension was measured using a wire myograph and representative myograph traces of tension are shown.

3.6 Changes in osmolality and osmolytes

Serum osmolality is regulated mainly by Na^+, K^+, BUN, and glucose. This regulation can be approximately expressed by the following formula: serum osmolality $= 2(Na^+ + K^+) +$ glucose/18 + BUN/2.8. To understand which component is the cause of the changes in serum osmolality in preterm infants, these osmolytes were measured in CB and blood samples after birth. Patients' profiles for osmolytes analysis are shown in Supplementary material online, *Table S2*. Changes in serum Na^+ concentration were significantly correlated with those in serum osmolality (*Figure 7A*). No correlation between serum osmolality and K^+, BUN, or glucose was observed (*Figure 7B–D*).

4. Discussion

We have demonstrated that serum osmolality decreases after birth and gradually increases over the next few days, even in moderate- and late-preterm human infants. These findings are similar to those reported in a study performed in the late 1960s, in which it was found that serum osmolality of normal full-term infants was depressed after birth and rose to normal adult levels by Day 5.[20] We have further demonstrated in this study that extremely preterm infants showed no such early decrease, but exhibited a rapid increase in serum osmolality. This was particularly so among those infants with PDA. Using rat DAs, we then provided evidence supporting the hypothesis that serum hypoosmolality promotes DA closure, and that the mechanism most likely involves the hypoosmolality sensor TRPM3. Accordingly, although serum osmolality *per se* has been largely ignored in the past few decades, it may play an important role in regulating DA closure.

Since hypoosmolality raises $[Ca^{2+}]_i$ in rat DASMCs and increases tension in the rat DA, the ability to sense a decrease in serum osmolality appears to be important for DA closure. We found that the hypoosmolality sensor TRPM3 was expressed in the DA tissues of humans and rats, and that TRPM3 is involved in hypoosmolality-induced DA contraction. TRPM3 is widely expressed in mammalian cells, including the collecting tubular epithelium of the kidney, pancreatic β-cells, neurons, and vascular SMCs.[31–34] Recently, Naylor *et al.*[32] reported the existence of a TRPM3 protein in the smooth muscle layer of the human saphenous vein and examined the functional role of TRPM3 in contractile responses in the mouse aorta. Majeed *et al.*[34] have also demonstrated TRPM3-mediated $[Ca^{2+}]_i$ increases in human vascular SMCs. These data are consistent with our finding that TRPM3 contributes to closure of the DA via increased $[Ca^{2+}]_i$. Although we have not examined whether a deficiency of TRPM3 would cause a failure of DA closure, the occurrence of PDA has not been reported in the studies on TRPM3 knockout mice.[35,36] In this context, the hypoosmolality-induced DA

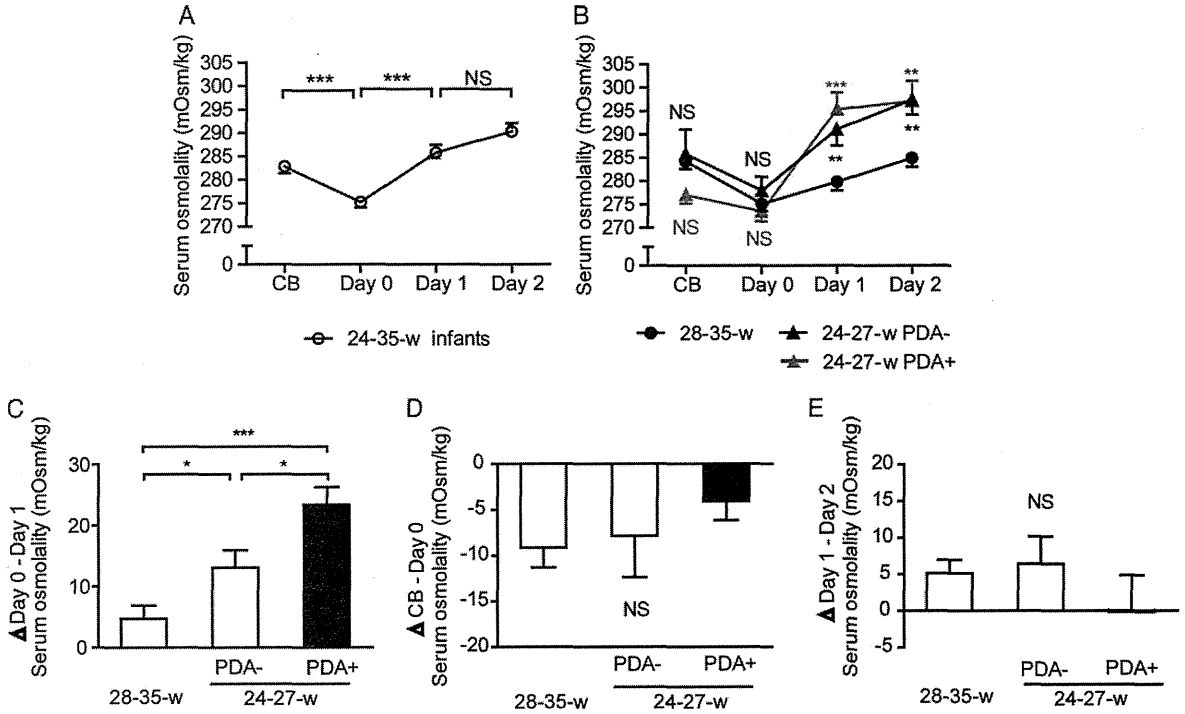

Figure 6 Changes in serum osmolality in human preterm infants. (*A*) Time course of serum osmolality in the entire preterm infant group born at 24- to 35-week gestation. $n = 62$, ***$P < 0.001$. NS: not significant. Osmolality was measured using a freezing-point depression osmometer. CB: cord blood. (*B*) Time courses of serum osmolalities in moderate- and late-preterm infants born at 28- to 35-week gestation ($n = 35$) and in extremely preterm infants born at 24- to 27-week gestation without PDA ($n = 12$) and with PDA ($n = 15$). **$P < 0.01$; ***$P < 0.001$; NS: not significant vs. 28- to 35-week gestation. (*C*) Increase (Δ) in serum osmolality from Day 0 to Day 1 in extremely preterm infants with PDA born at 24- to 27-week gestation was larger than that in those without PDA at the same gestational age or moderate- and late-preterm infants born at 28- to 35-week gestation. *$P < 0.05$; ***$P < 0.001$. (*D* and *E*) Changes from CB to Day 0 (*D*), and from Day 1 to Day 2 (*E*). NS: not significant.

closing mechanism, unlike the well-known oxygen-induced constriction mechanism, may not be the principal mechanism for DA closure. In addition to hypoosmolality-induced DA closure, our data suggested that *hyper*osmolality attenuates DA closure in a TRPM3-independent manner. Although previous reports demonstrated that hypertonic stimulation induced arterial dilatation via activation of potassium channels,[37,38] whether *hyper*osmolality-induced activation of potassium channels also promotes dilatation in the DA needs to be examined in the future.

Accumulating evidence suggests that TRPM3 could be regulated by pregnancy hormones and by the pharmacological treatment of PDA.[34,39] TRPM3 is known to be stimulated by physiological steroids including pregnenolone sulfate.[30] Majeed *et al.*[34] demonstrated that progesterone bonded to TRPM3 and inhibited endogenous TRPM3 activity in human vascular SMCs. It is of interest that foetuses are exposed to high levels of progesterone during the course of pregnancy, and that rapid withdrawal of this hormone occurs upon separation from the placenta.[40] Because our data demonstrated that TRPM3 mRNA was highly expressed in both premature and mature rat foetuses, it is tempting to speculate that TRPM3 might be kept inactive until birth by placental progesterone.

The mechanism by which serum osmolality is decreased at birth remains to be elucidated. However, two mechanisms, at least, may be considered. Serum osmolytes may be withdrawn through utilization and/or excretion via the kidneys at birth. Alternatively, osmolytes may be diluted in circulating blood. In this regard, we found that changes in serum osmolality were best correlated with those of Na^+ among other osmolytes, such as K^+, glucose, or BUN. Because serum Na^+ may not be utilized or excreted rapidly within an hour after birth, it is most likely that dilution of the circulating blood occurs. In the present study, there was no significant correlation between K^+, glucose, or BUN and osmotic changes. However, the data, which included a relatively small number of samples, did not negate the contributions of small amounts of K^+, glucose, and BUN to osmotic changes. This explanation agrees, at least in part, with previous studies demonstrating increased lung fluid clearance at respiratory onset: it has been shown that, immediately after birth, lung fluid shifts to the circulating blood compartment via aquaporins in the lung epithelium.[41,42] This may cause dilution of circulating blood, although lung fluid is also expelled mechanically at birth. The exact mechanisms of the transient decrease in serum osmolality, however, need to be further examined in future studies.

Our findings may also be of some practical use to paediatricians treating patients with PDA, i.e. serum osmolality should not be allowed to rise too high among extremely preterm infants with PDA. In general, such infants are likely exposed to increased serum osmolality. Because paediatricians are afraid of fluid overload and thus worsening of the patency of the DA simply due to circulating volume expansion,[43,44]